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13. ABSTRACT (Maximum 200 Words) <p>Signal regulatory proteins (SIRPs include SIRPβ1, which activates cells, and SIRPα1, which inhibits the cellular response to several growth factors, and which regulates cell adhesion and spreading.</p> <p>We demonstrated by PCR that 3 of 3 prostate cancer cell lines (PC-3, DU-145 and LNCaP) express transcripts for SIRPs. Under this contract, we generated a monoclonal antibody that recognizes both SIRPβ1 and SIRPα1, thereby confirming the expression of SIRPs on PC-3 cells and, to a lesser extent on DU-145 cells. The receptor could not be detected on LNCaP cells. We have since shown by PCR, Western blotting, and by surface staining that PC-3 and DU-145 cells express SIRPα1 but not SIRPβ. We find that they also express the tyrosine phosphatase, SHP-2, and that SHP-2 binds to SIRPα1 when it is phosphorylated, demonstrating that this pathway for the function of SIRPα1 is intact. We have created constructs of epitope-tagged SIRPα1, either intact or with mutations that would alter SHP-2 binding, in order to study its function in PC-3 cells.</p> <p>We have also worked in particular on the characterization of the SIRPα1 protein in prostate cancer cells. Is there more than one form, due either to alternate splicing or to post-translational modification? These studies have proved challenging, but we expect to complete them, along with all of the objectives of the contract, over the coming year (no-cost extension).</p>				
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INTRODUCTION

Signal regulatory proteins (SIRPs, also known as SHPS-1, BIT, p84, and Myd-1) are normally expressed on certain hematopoietic cells and some brain cells (1-3). SIRP β 1 activates cells and is expressed on cells of monocyte/macrophage lineage. Its ligand is unknown. SIRP α 1 inhibits the response of several cell types to growth factors (1), and it regulates integrin-mediated cell adhesion and spreading (4,5). Its ligand is CD47 (integrin associated protein)(6,7). SIRP β 1 and SIRP α 1 are highly homologous in their extracellular portions, which include three immunoglobulin (Ig)-like domains (one V and two C domains). By alternative splicing, SIRP α 1 can also be expressed with a single Ig-like (V) domain (8). Phosphorylated SIRP α 1 binds SHP-2, a tyrosine phosphatase that is widely distributed (1). Thus, expression of SIRPs on tumor cells might be functional and could regulate the response to growth factors and/or the capacity of tumors to invade.

Our Studies were based on our identification by PCR of transcripts for signal regulator protein α 1 (SIRP α 1) in three prostate cancer cell lines, PC-3, LNCaP, and DU-145. Because of the role of SIRP α 1 in regulating the response to growth factors and of other functions that may affect the malignant potential of prostate cancer, we proposed to further examine the expression and function of SIRPs in these prostate cancer cells. To this end, we proposed six objectives:

1. Examine SIRP transcript size and expression level in different prostate cancer cell lines by Northern blotting.
2. Use RT-PCR to obtain and to sequence full-length SIRP transcripts from the prostate cancer cell lines PC-3, DU-145, and LNCaP.
3. Use hybridization to screen cDNA libraries from these prostate cancer cell lines to identify additional SIRP cDNA clones.
4. Overexpress wild-type and mutated SIRPs in prostate cancer lines and, as a control, in NIH3T3 fibroblasts, to assess their effect on the cellular growth response to EGF. These will include studies of the effect of cross-linking SIRPs in cell growth. Mutational studies will include: for alpha SIRPs, mutate the cytoplasmic tyrosines to phenylalanine, for beta SIRPs, mutate the transmembrane lysine to alanine.
5. Assess the effect of EGF on the phosphorylation of SIRPs in prostate cancer cells and of associated proteins obtained by co-immunoprecipitation.
6. Produce monoclonal antibodies (mAbs) against SIRPs.

BODY

This is a revised final report. The prior review of this report requested resubmission with additional data to demonstrate that we had adequately tested the hypotheses. In discussion of the report with Dr. Nrusingha Mishra, it was pointed out that this was in

part necessary because our studies had not produced publishable results. The reviewer noted that our basic hypothesis has proven to be wrong, but the results are again briefly summarized below in the event that there are new reviewers, and to provide some additional comments.

Our studies confirmed the expression of SIRP α in prostate cancer cell lines, particularly PC-3 cells, and they demonstrated that SIRP α in these cells is active in that it binds the tyrosine phosphatase SHP-2. Heartened by these results, we extended our studies beyond the proposed work in cancer cell lines, to define the expression of SIRPs in prostate cancer biopsies. These studies were done in collaboration with Dan Sudilovsky, M.D., a pathologist at UCSF who specializes in the pathology of the prostate. Using our monoclonal antibody to SIRPs (derived as part of this grant) in immunohistochemical studies, we unfortunately could not confirm the expression of SIRPs in freshly obtained biopsies of prostate cancer. In light of these negative findings, we have not yet considered our studies of prostate cancer cell lines worthy of publication beyond the AACR abstract in which they were initially announced.

We also, however, gave the cDNAs for both SIRP α 1 and for SIRP β 1 to Chris Haqq, M.D., at UCSF, and the expression of SIRPs has been included in the ongoing studies at UCSF of gene expression in prostate cancer. Per Dr. Haqq, the signal strength from SIRPs is low in biopsies of both prostate and prostate cancer, and if there is any difference between them (which is not yet clear), it is lower in cancer. The UCSF group, however, is also comparing primary prostate cancer to metastatic prostate cancer. These studies are at an early stage (9). It may be of note that the PC-3 prostate cancer line, in which SIRP α 1 is most prominently expressed, was derived from a prostate adenocarcinoma that was metastatic to bone (10). Also, as noted in our original application, a sequence segment with homology to SIRP α 1 has been identified in a specimen of prostate cancer that was metastatic to bone (GenBank accession AA937670). Thus, we are following with interest the possibility that SIRPs might be selectively expressed in some metastases of prostate cancers or in invasive cancer. If this proves to be the case, it will give new importance to our studies of SIRPs in cell prostate cancer cell lines.

In the prior report, I failed to include a publication from my lab regarding the expression of SIRPs in glial tumors (11). These studies made use of our monoclonal antibody to SIRPs, and the approach to these studies derived from our studies of SIRP expression in prostate cancer cell lines. The publication therefore made appropriate reference to partial support from our DoD grant, although the primary support was from a grant from UCSF. I have appended a copy of this publication. In these studies, we were able to demonstrate SIRP expression not only on astrocytoma cell lines but also, by immunohistochemistry, in fresh specimens of glial tumors. These findings gave us additional confidence that our inability to detect SIRP expression in biopsies of prostate cancer was because SIRPs were not expressed at appreciable levels in these specimens. These studies also demonstrate the potential usefulness of our antibody, and we have made it freely available.

In the prior report, the results of our studies were presented chronologically. We have here presented them instead in the order of the specific aims, in order to facilitate review of each, though we have retained the list of accomplishment by the year in which they were obtained. I have included figures in particular for recent work. I have not included the immunohistochemical stains of prostate cancer biopsies because these were negative.

Specific Aim 1. Examine SIRP transcript size and expression level in different prostate cancer cell lines by Northern blotting. Completed. Although transcripts for SIRPs could be detected in PC-3, DU-145, and LNCaP cells by RT-PCR, the expression in LNCaP cells was very weak, and we could not detect it by Northern blotting. These results were confirmed by flow cytometric analysis of SIRP expression using our monoclonal antibody, produced as part of Specific Aim 6; SIRPS could be detected on PC-3 cells, to a lesser extent on DU-145 cells and not on LNCaP cells, as shown in Fig. 1.

Specific Aim 2. Use RT-PCR to obtain and to sequence full-length SIRP transcripts from the prostate cancer cell lines PC-3, DU-145, and LNCaP. Largely completed. Because partial sequences from the three cell lines were identical, we focused these studies on PC-3 cells. We obtained sequence from multiple PCR clones derived by using different PCR primers. All of these sequences indicate that the expressed transcript is encoded by SIRP α 1, not SIRP β , but many sequences were fragmented and some had alternate starts. In none have we been able to sequence in a single run from the 3' end to the 5' end, because there is a block to sequencing at the 5' end that we have not been able to overcome. Therefore sequences are derived from overlapping RT-PCRs from the same clone. Some of the derived protein sequences are shown in Fig. 2, where they are compared to the sequences for SIRP α 1 and SIRP β 1. The most complete clone (hcp1607188b) is almost identical to SIRP α 1 except that it has an alternate start site, adding 22 residues, but many other partial clones use the published start site. We conclude that PC-3 cells express SIRP α 1 and not SIRP β 1, but that alternate forms of SIRP α 1 may be expressed.

Specific Aim 3. Use hybridization to screen cDNA libraries from these prostate cancer cell lines to identify additional SIRP cDNA clones. Completed through alternate approaches. In collaboration with John Kokontis at the University of Chicago, we screened his cDNA library from PC-3 cells. We obtained no clones and we subsequently confirmed by both RT-PCR and flow cytometry that his subclone of PC-3 cells has lost the expression of SIRPs. With the publication of the human genome, it was revealed that there is only one SIRP α 1 gene within the SIRP family locus on chromosome 20. As discussed in our attached paper, there is also a second SIRP α gene on chromosome 22 that is encoded as a single exon, but it has not yet been demonstrated that this gene is expressed. We have therefore not further pursued the use of libraries to identify alternate forms of SIRP α 1 in addition to those identified by PCR, using primers from different areas of the gene.

Specific Aim 4. Overexpress wild-type and mutated SIRPs in prostate cancer lines and, as a control, in NIH3T3 fibroblasts, to assess their effect on the cellular growth response to EGF. These will include studies of the effect of cross-linking SIRPs in cell growth. Mutational studies will include: for alpha SIRPs, mutate the cytoplasmic tyrosines to phenylalanine, for beta SIRPs, mutate the transmembrane lysine to alanine. Completed, but unsuccessfully. For these studies, we mutated the cDNA for SIRP α 1, changing the tyrosines in both ITIMs (immunoreceptor tyrosine-based inhibitory motifs) to phenylalanines (residues 465 and 495). Both the intact and mutated receptor were expressed in PC-3 cells, appending a FLAG epitope to the 5' end to allow their identification and their ligation by anti-FLAG antibody without ligating the endogenous SIRP α 1 receptors. The wild type and mutated receptor were expressed at comparable levels. We could not, however, express the effect of these receptors on the response of the PC-3 cells to EGF because we could not demonstrate a change in the growth of PC-3 cells in response to EGF. These studies included varying periods of resting cells in serum-free medium prior to stimulation. There have been conflicting reports on the capacity of EGF to stimulate growth of PC-3 cell, but there is increasing evidence against responsiveness to EGF in the absence of androgens (12-15). We find the same. An example is shown in Fig. 3. Overexpression of intact SIRP α 1 reduced the basal level of proliferation of PC-3 cells, although inconsistently, and the cells remained unresponsive to EGF. Overexpression of mutated SIRP α 1 affected neither the basal level of PC-3 proliferation nor its response to EGF. Ligation of the receptor with antibodies to FLAG had no effect on the growth response of PC-3 cells to EGF. Because of these results, we have not further pursued this specific aim.

Specific Aim 5. Assess the effect of EGF on the phosphorylation of SIRPs in prostate cancer cells and of associated proteins obtained by co-immunoprecipitation. Alternate studies pursued and completed. Because our PC-3 cells proved unresponsive to EGF, we added an alternative approach to demonstrate the functional association of SIRP α 1 with signaling molecules. In hematopoietic cells ITIMs recruit the tyrosine phosphatases SHP-1 and SHP-2, and SIRP α 1 has been shown to associate with SHP-1, which is the more abundant. The expression of SHP-1, however, is restricted to hematopoietic cells, while that of SHP-2 is not. We were therefore interested to determine if SIRP α 1 in PC-3 cells could associate with SHP-2. To test this, PC-3 cells were exposed to pervanadate, an inhibitor of tyrosine phosphatase, in order to increase phosphorylation of SIRP α 1. Cell lysates were then immunoprecipitated with an antibody to SHP-2 (transduction laboratories) under conditions that favor co-immunoprecipitation of associated molecules. The immunoprecipitate was resolved by SDS-PAGE and examined for SIRP α 1 by Western blotting with our mAb to SIRP α 1. As controls, we examined CHO cells transfected with the cDNA for SIRP α 1. As shown in Fig. 4, immunoprecipitation of SHP-2 from PC-3 also precipitates SIRP α 1. From these results, we conclude that SIRP α 1 in PC-3 cells is functional to the extent that it can recruit SHP-2.

Specific Aim 6. Produce monoclonal antibodies (mAbs) against SIRPs. Completed. We found that this antibody recognizes both SIRP α and SIRP β . The use of this

antibody is demonstrated in our Western blots and in flow cytometry. It is also used in the attached paper.

Additional studies. Some of our additional studies have been noted above, including the use of our mAb to demonstrate the expression of SIRP α 1 on PC-3 cells. We considered the possibility, however, that our antibody was recognizing something other than SIRP α 1 on the PC-3 cells, and so we also used immunoprecipitation followed by Western blotting to assess the expression of SIRP α 1 on PC-3 cells, with comparison to CHO cells that had been transfected with SIRP α 1 (CHO- SIRP α 1). Our mAb recognizes SIRPs broadly, and we wanted specifically to exclude recognition of SIRP β (even though we could not find message for SIRP β). We therefore used a commercial antiserum to the cytoplasmic domain of SIRP α 1, so that we could immunoprecipitate with this and then blot with our mAb to SIRPs. (SIRP β 1 has almost no cytoplasmic domain.) This proved to be a very challenging task, and it was complicated by the fact that SIRPs in both PC-3 cells and CHO- SIRP α 1 cells are variably glycosylated, yielding isoforms that were not distinctly resolved and that differed in size between the two lines. We therefore added a step in which the immunoprecipitates were deglycosylated before electrophoresis and Western blotting. The SIRP α 1 in CHO- SIRP α 1 cells was partially resistant to this step, perhaps because of its abundance, but eventually we were able to confirm that both PC-3 cells and CHO- SIRP α 1 cells expressed a protein of the same size (and the appropriate size for SIRP α 1), as shown in Fig. 5.

We hope that this revised report makes evident the extensive amount of work that we have put into this project, extending beyond our original specific aims.

KEY RESEARCH ACCOMPLISHMENTS

Year 1

1. The production of monoclonal antibodies to SIRPs
2. The use of monoclonal antibodies to confirm the expression of SIRPs on prostate cancer cells.
3. Stable overexpression of SIRP α 1 and of SIRP β 1 in PC-3 cells.

Year 2

1. Confirmation of SIRP transcripts in prostate cancer cells by Northern blotting.
2. Confirmation of SIRP α 1 transcripts in PC-3 cells by PCR (no evidence for SIRP β).
3. Confirmation by Western blotting that SIRP α is expressed in PC-3 cells
4. Demonstration that PC-3 cells express the SIRP substrate SHP-2
5. Demonstration in PC-3 cells of the interaction of SIRP with SHP-2.

Year 3

1. Resolution of SIRP α protein size, expressed in prostate cancer cells in both glycosylated and deglycosylated forms.
2. Demonstration by flow cytometry that prostate cancer cells do not express SIRP β .
3. Construction of mutant SIRP α 1, lacking the cytoplasmic tyrosine required for the recruitment of SHP-2.

Year 4

1. Mutation of SIRP α 1 at tyrosine residues that are required for the recruitment of the tyrosine phosphatase SHP-2.
2. Expression of intact and mutated SIRP α 1 in PC-3 cells
3. Studies (negative) of the effects of SIRP α 1 on the growth response to EGF, whether SIRP α 1 is ligated or not.

REPORTABLE OUTCOMES

See Conclusions.

CONCLUSIONS

Our studies confirm that PC-3 cells express SIRP α 1, and that phosphorylation of this receptor leads to its association with the tyrosine phosphatase, SHP-2. We have not, however, been able to discern a functional effect of SIRP α 1 on the response of these cells to EGF, and the importance of our finding in prostate cancer cells is diminished by the growing evidence that expression of SIRP α 1 is not increased in fresh specimens of prostate cancers. Thus, while we could report our findings in PC-3 cells, we are not, in the end, inclined to assert that this has importance in prostate cancer, unless it proves that SIRP α 1 is differentially expressed in metastases of prostate cancer or in invasive tumor specimens.

On the plus side, our work has provided a series of reagents that are available to the scientific community, and our work led a young investigator in our lab, Tom Chen, to study the expression of SIRPS in brain cancer. Here the evidence supports a role for SIRPs in cancer (11). Dr. Chen has received a Career Development Award from the Veterans Administration to pursue this work. Thus, some good has come of our studies, though not the good that we expected.

REAGENTS DEVELOPED

Notable among these are:

1. Mouse monoclonal antibody to human SIRPs, cross-reactive with both SIRP α and SIRP β .
2. cDNA for SIRP α 1, mutated to inactivate both ITIMs.
3. Cell lines (PC-3, CHO) expressing transfected SIRP α 1 or SIRP β 1, both FLAG-tagged.

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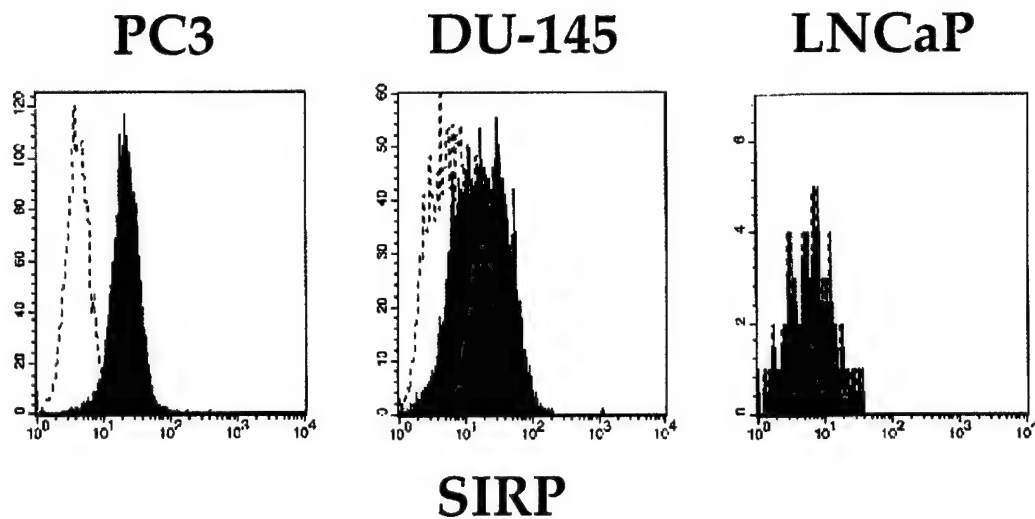


Figure 1. SIRP expression on prostate carcinoma cell lines as assessed by flow cytometry with our anti-SIRP mAb (followed by a second-step anti-mouse antiserum). The open line shows staining with second-step antiserum alone. The filled line shows staining with anti-SIRP. The results demonstrate that SIRPs are expressed on the surface of PC-3 cells. They are also expressed on the surface of DU-145 cells, although the staining is broader in distribution compared to PC-3 cells, and the background for DU-145 cells is higher, reducing discrimination between stained and unstained cells, SIRPs could not be detected on LNCaP cells. These results correlate with levels of message for SIRPs, as assessed either by RT-PCR or by Northern blotting.

Figure 2. Predicted peptide sequences from 5 independently-derived cDNAs, obtained by RT-PCR from PC-3 cells, using primers derived from SIRPs. The sequences are compared to the predicted sequence for (top row) and for SIRPa1 (bottom row).

SIRPa1-Ullrich	1	60
hclp1607188b	---	--MEPAGPAP GRGGLICLL LAASCAWSGV
hclp1628334	---	---MAGGARGPVP GRGGLICLL LPASCAWSGV
hclp1628344b	---	---MAGGARGPVP GRGGLICLL LPASCAWSGV
hclp1628344b	---	---MAGGARGPVP GRGGLICLL LPASCAWSGV
hclp49210	---	---MAGGARGPVP GRGGLICLL LPASCAWSGV
hclp49218	---	---MAGGARGPVP GRGGLICLL LPASCAWSGV
SIRPB1-Ullrich	---	---MAGGARGPVP GRGGLICLL LPASCAWSGV
SIRPa1-Ullrich	1	160
hclp1607188b	---	---MAGGARGPVP GRGGLICLL LPASCAWSGV
hclp1628334	---	---MAGGARGPVP GRGGLICLL LPASCAWSGV
hclp1628344b	---	---MAGGARGPVP GRGGLICLL LPASCAWSGV
hclp49210	---	---MAGGARGPVP GRGGLICLL LPASCAWSGV
hclp49218	---	---MAGGARGPVP GRGGLICLL LPASCAWSGV
SIRPB1-Ullrich	---	---MAGGARGPVP GRGGLICLL LPASCAWSGV
SIRPa1-Ullrich	1	260
hclp1607188b	---	---MAGGARGPVP GRGGLICLL LPASCAWSGV
hclp1628334	---	---MAGGARGPVP GRGGLICLL LPASCAWSGV
hclp1628344b	---	---MAGGARGPVP GRGGLICLL LPASCAWSGV
hclp49210	---	---MAGGARGPVP GRGGLICLL LPASCAWSGV
hclp49218	---	---MAGGARGPVP GRGGLICLL LPASCAWSGV
SIRPB1-Ullrich	---	---MAGGARGPVP GRGGLICLL LPASCAWSGV
SIRPa1-Ullrich	1	360
hclp1607188b	---	---MAGGARGPVP GRGGLICLL LPASCAWSGV
hclp1628334	---	---MAGGARGPVP GRGGLICLL LPASCAWSGV
hclp1628344b	---	---MAGGARGPVP GRGGLICLL LPASCAWSGV
hclp49210	---	---MAGGARGPVP GRGGLICLL LPASCAWSGV
hclp49218	---	---MAGGARGPVP GRGGLICLL LPASCAWSGV
SIRPB1-Ullrich	---	---MAGGARGPVP GRGGLICLL LPASCAWSGV

SIRPAL-ullrich	VKLTCQVEHD	GQPAVSKSHD	LKVSHPKEQ	GSNTAENTG	SNERNIYI..	VGVVCTLLV	ALLMAALYIV	RIRQKAQGS	TSSTRLHEPE	KNAREITQDT
hcp1607188b	VKLTCQVEHD	GQSAVSKSHD	LKVSAHLKEQ	SSNTAAENTG	PNEQNIYI..	VGVVCTLLV	ALLMEALYIV	RIRQKAQGS	TSSTRLHEPE	KNARKITQDT
hcp1628334										
hcp1628344b										
hcp49210	GQPAVSKSHD	LKVSHPKEQ	GSNTAENTG	SNERNIYI..	VGVVCTLLV	ALLMAALYIV	RIRQK.....
hcp49218										
SIRPB1-ullrich	LVNTPCAHRDD	VWLTQCV EHD	GQQAVSKSYA	LEISAHQEH	GSDITHEPAL	APTAPILV..	ALLGP KILL	VGVSAIYIC	WKOKA~	

	461	540
SIRP1-ullrich	NDITVADLNL PKGKKPAPQA AEPNNHTEYA SIQTSQPQAS EDTLITYADLD MVHLNRTPKQ PAPKEPSFS EYASVQVPRK	
hcp1607188b	NDITVADLNL PKGKKPAPRA AEPNNHTEYA SIQTSQPQAS EDTLITYADLD MVHLNRTPKQ LAPKEPSFS EYASIQVPRK	
hcp1628334		
hcp1628344b		
hcp49210DLNL PKGKKPAPQA AEPNNHTEYA SIQTSQPQAS EDTLITYADLD MVHLNRTPKQ PAPKEPSFS EYASVQVPRK	
hcp49218		
SIRPB1-ullrich		

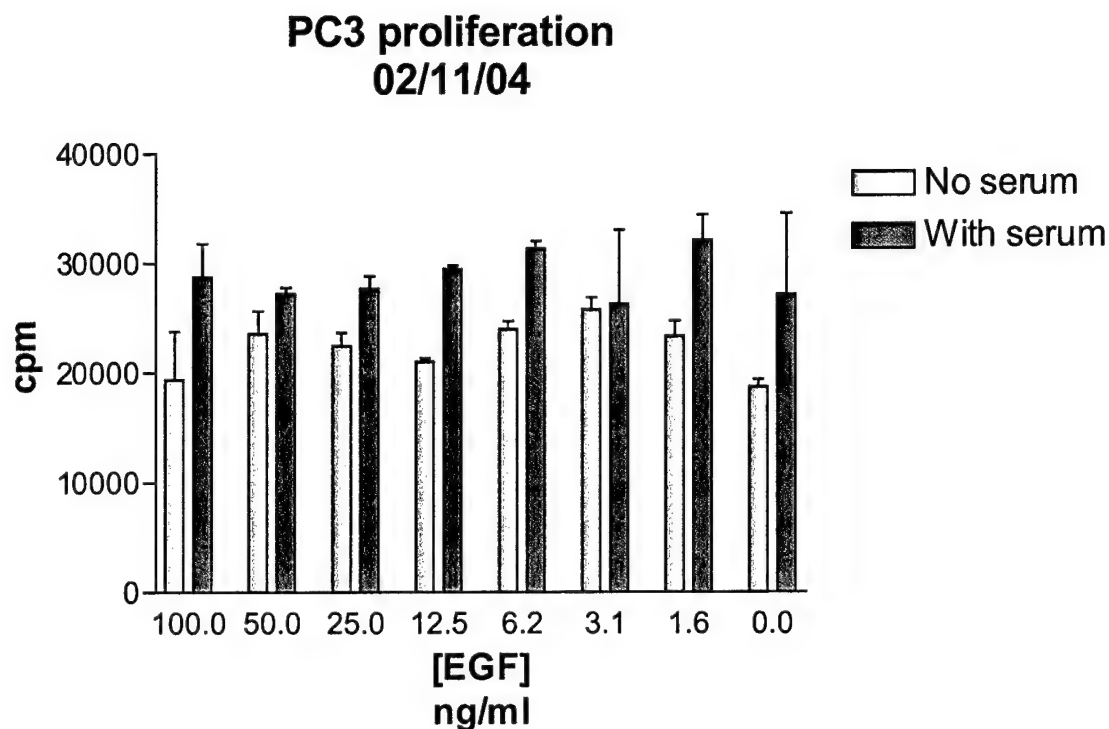


Figure 3. EGF does not alter the proliferation of PC-3 cells grown either in serum or without serum (after serum-starvation for 24 hrs. PC-3 cells were cultured with or without serum in the presence of EGF at the concentrations shown. In this experiment, ^3H -thymidine was added at the onset, and uptake into cells was assessed 18 hr later.

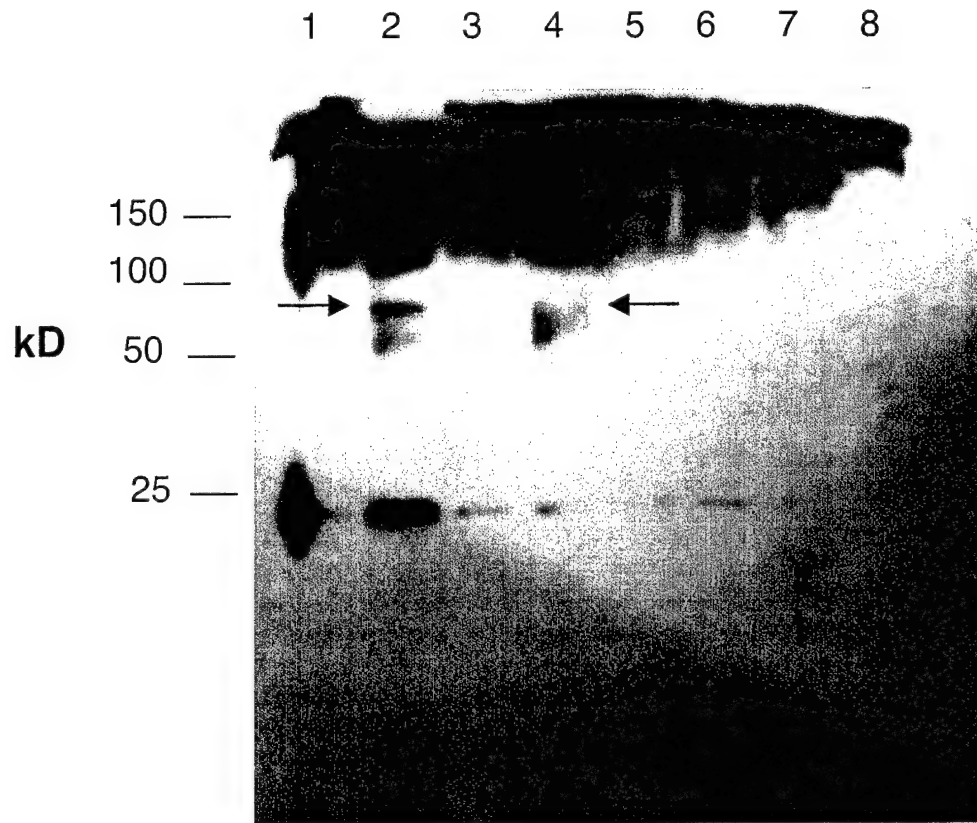


Figure 4. Co-immunoprecipitation of SIRP from PC-3 cells by antibody to SHP-2. SIRP is identified after SDS-PAGE by Western blotting with monoclonal antibody to SIRP. Lane 1 = unstimulated PC-3 cells. Lane 2 = pervanadate-stimulated PC-3 cells. Lane 3 = unstimulated CHO-SIRP α 1 cells. Lane 4 = pervanadate-stimulated CHO-SIRP α 1 cells. Lane 5 = unstimulated CHO-SIRP β 1 cells. Lane 6 = pervanadate-stimulated CHO-SIRP β 1 cells. Lane 7 = unstimulated CHO cells (untransfected). Lane 8 = pervanadate-stimulated CHO cells (untransfected). Right arrow indicates blotting of SIRP in SHP-2 precipitates from pervanadate-treated PC-3 cells. Left arrow indicates blotting of SIRP in SHP-2 precipitates from pervanadate-treated CHO-SIRP α 1 cells. No blotting for SIRP was seen in pervanadate-treated CHO-SIRP β 1 cells, in pervanadate-treated CHO cells (untransfected), or in any cells that were unstimulated by pervanadate. The identity of the band below 25 kD, found in all cells, is unknown.

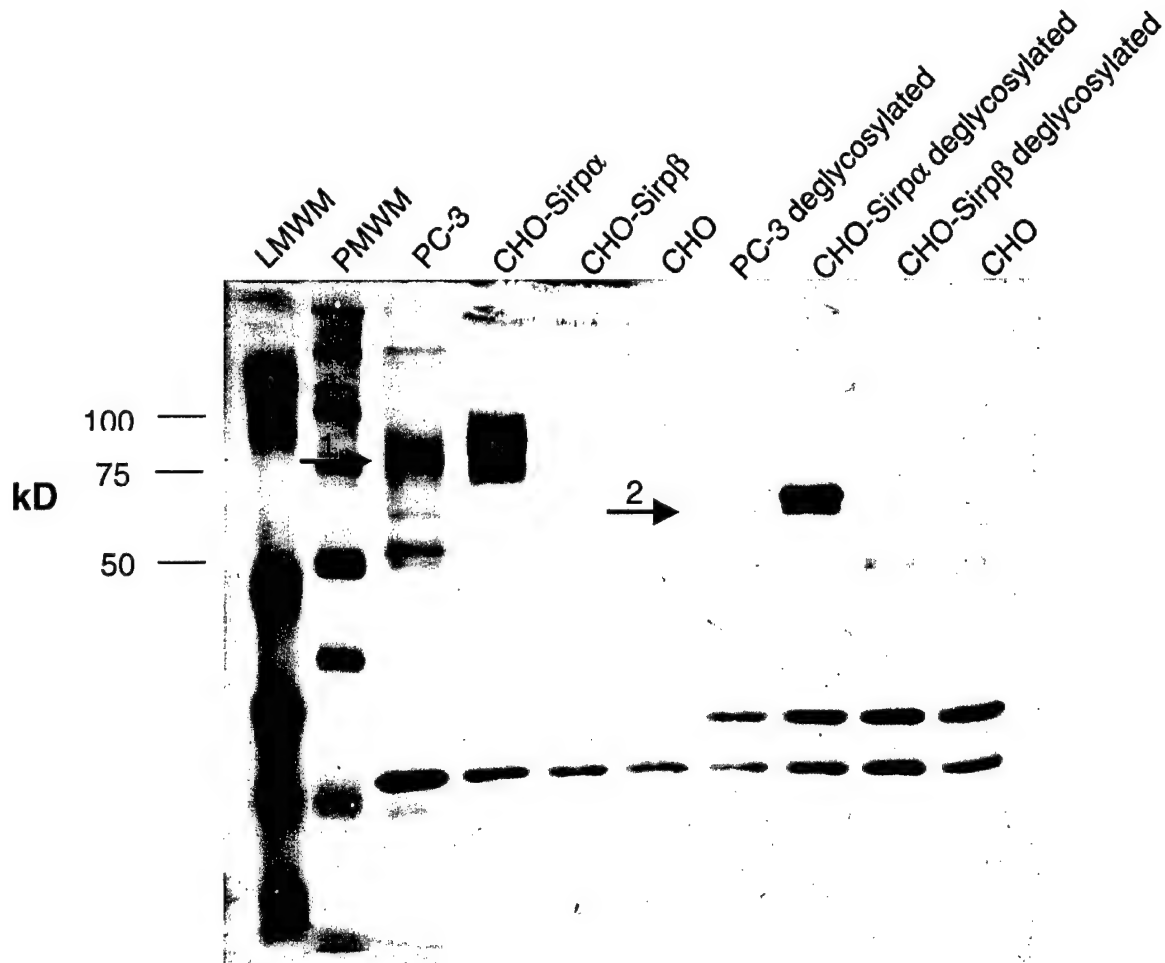


Figure 5. Immunoprecipitation of glycosylated and deglycosylated SIRP α from PC-3 cells, compared to CHO cells transfected with SIRP α 1 (or with SIRP β 1 and with untransfected CHO cells). Cell lysates were first immunoprecipitated with a commercial antiserum to the cytoplasmic domain of SIRP α 1. Cell lysates were resolved by SDS-PAGE and subjected to Western blotting with our mAb to SIRPs. Arrow 1 shows glycosylated SIRP precipitated from PC-3 cells, compared to CHO-SIRP α 1 cells in the next lane. Although both bands are broadly distributed, the SIRP from PC-3 cells runs below that from CHO-SIRP α 1. Arrow 2 shows deglycosylated SIRP from PC-3 cells. Although its intensity is not well seen on this print, it is more clearly seen on the original blot. It runs level with a band of deglycosylated SIRP α 1 from CHO-SIRP α 1 cells. There is also a more intense band just above this. We suspect, but cannot prove, that this is SIRP α 1 that has not been completely deglycosylated.

Expression and Activation of Signal Regulatory Protein α on Astrocytomas

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ABSTRACT

High-grade astrocytomas and glioblastomas are usually unresectable because they extensively invade surrounding brain tissue. Here, we report the expression and function of a receptor on many astrocytomas that may alter both the proliferative and invasive potential of these tumors. Signal regulatory protein (SIRP) α 1 is an immunoglobulin superfamily transmembrane glycoprotein that is normally expressed in subsets of myeloid and neuronal cells. Transfection of many cell types with SIRP α 1, including glioblastomas, has been shown to inhibit their proliferation in response to a range of growth factors. Furthermore, the expression of a murine SIRP α 1 mutant has been shown to enhance cell adhesion and initial cell spreading but to inhibit cell extension and movement. The extracellular portion of SIRP α 1 binds CD47 (integrin-associated protein), although this interaction is not required for integrin-mediated activation of SIRP α 1. On phosphorylation, SIRP α 1 recruits the tyrosine phosphatases SHP-1 and SHP-2, which are important in its functions. Although SHP-1 is uniquely expressed on hematopoietic cells, SHP-2 is ubiquitously expressed, so that SIRP α 1 has the potential to function in many cell types, including astrocytomas. Because SIRP α 1 regulates cell functions that may contribute to the malignancy of these tumors, we examined the expression of SIRPs in astrocytoma cell lines by flow cytometry using a monoclonal antibody against all SIRPs. Screening of nine cell lines revealed clear cell surface expression of SIRPs on five cell lines, whereas Northern blotting for SIRP α transcripts showed mRNA present in eight of nine cell lines. All nine cell lines expressed the ligand for SIRP α 1, CD47. To further examine the expression and function of SIRPs, we studied the SF126 and U373MG astrocytoma cell lines, both of which express SIRPs, in greater detail. SIRP transcripts in these cells are identical in sequence to SIRP α 1. The expressed deglycosylated protein is the same size as SIRP α 1, but in the astrocytoma cells, it is underglycosylated compared with SIRP α 1 produced in transfected Chinese hamster ovary cells. It is nonetheless still capable of binding soluble CD47. Moreover, SIRP α 1 in each of the two cell lines recruited SHP-2 on phosphorylation, and SIRP α 1 phosphorylation in cultured cells is CD47 dependent. Finally, examination of frozen sections from 10 primary brain tumor biopsies by immunohistochemistry revealed expression of SIRPs on seven of the specimens, some of which expressed high levels of SIRPs. Most of the tumors also expressed CD47. This is the first demonstration that astrocytomas can express SIRP α . Given the known role of SIRP α in regulating cell adhesion and responses to mitogenic growth factors, the expression of SIRP α 1 on astrocytomas may be of considerable importance in brain tumor biology, and it offers the potential of a new avenue for therapeutic intervention.

INTRODUCTION

Signal regulatory proteins (SIRPs) comprise a receptor family whose structure includes three extracellular immunoglobulin loop (immunoglobulin) domains (reviewed in Refs. 1 and 2). Orthologs of human SIRP α 1 are expressed in rats (SHPS-1, MFR, and p93), mice (p84 and BIT), and cattle (MyD-1). SIRP α receptors have a cytoplas-

mic tail that includes several immunoreceptor tyrosine-based inhibitory motifs (ITIMs). Phosphorylation of the tyrosines in these motifs leads to the recruitment and activation of tyrosine phosphatases SHP-1 and SHP-2 (3, 4). SIRP β receptors have only a short cytoplasmic tail, with no ITIMs. Instead, they express a charged residue in the transmembrane domain, which permits association with the adapter protein DAP12 (5, 6). SIRP β receptors have been identified only in cells of the monocyte, macrophage, or dendritic lineages. Although initial reports using tissue Northern blotting suggested that SIRP α receptors are expressed ubiquitously (4), studies of protein expression suggest expression primarily in myeloid cells, subsets of brain cells (7, 8), and endothelial cells (9, 10). In the central nervous system, SIRP α receptors are expressed in the granular and molecular layers of the cerebellum as well as in various regions of the hippocampus, retina, and olfactory bulb (11-13). During development, SIRP α is expressed by embryonic day 9 on the floor plate region of the ventral neuraxis, and it is rapidly up-regulated by postnatal days 2-5 (14, 15). Examination of brain cell subtypes has established that SIRP α is expressed on cortical neurons as well as microglia, whereas neonatal astrocytes or cultured oligodendrocytes do not express detectable SIRP α (7).

A ligand for SIRP α is CD47, as demonstrated by the binding of soluble SIRP α fusion proteins to CD47 (10, 16-19) and binding of soluble CD47 to SIRP α expressed on myeloid cells (20, 21). In the mouse retina, the expression of CD47 and SIRP α is colocalized, and CD47-deficient mice show an altered pattern of SIRP α expression, especially in the cellular and plexiform layers of the retina, suggesting a functional association between the two molecules (12, 16). In rats, SIRP α expressed in neuronal cells demonstrates reduced glycosylation compared with SIRP α from myeloid cells, resulting in altered binding affinity to tissue sections or plant lectins (13, 22, 23), and SIRP α on neuronal cells exhibits differential glycosylation during embryonic development (15). Evidence of differential glycosylation is also seen in the MM5/C1 mouse mammary carcinoma and the A431 human epidermal carcinoma cell lines (4), and rat SIRP α expressed on peritoneal macrophages exhibits reduced glycosylation compared with SIRP α on alveolar macrophages (24). The functional significance of this altered glycosylation is unknown, but it might alter the avidity of SIRP α 1 for CD47, its isoforms, or other unknown ligands.

The initial characterization of human SIRP α cDNAs revealed several different sequences. For most SIRP α cDNAs, these differences clustered in the membrane distal immunoglobulin region (the V region), although for one cDNA (SIRP α 3), the sequence differences were distributed throughout the coding sequence (4). It appears likely that at least two cDNA variants for SIRP α (named SIRP α 1 and SIRP α 2 in the initial report above) are alleles of the same locus. The gene for SIRP α 1 can undergo alternative splicing, producing a low abundance cDNA that excludes the second and third immunoglobulin-like domains, and there are two polyadenylation sites that can yield the two predominant transcript sizes seen on Northern blotting (11, 25).

SIRP α is phosphorylated on its ITIMs in response to a variety of mitogenic growth factors [epidermal growth factor (EGF), platelet-derived growth factor, insulin, neurotrophins, growth hormone], cytokines (interleukin 1 β and tumor necrosis factor α), and lysophos-

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phatidic acid or after integrin-mediated adhesion to extracellular matrix components (reviewed in Refs. 1 and 2). In the case of adhesion, this phosphorylation is dependent on Src family kinases (26–28), but the kinases responsible for mitogen-stimulated phosphorylation of SIRP α remain unclear. In addition to being phosphorylated by these stimuli, SIRP α down-regulates responsiveness to EGF (4), growth hormone (29–31), insulin-like growth factor (4, 32), serum (3, 4, 33), neurotrophins (34, 35), and lysophosphatidic acid (36). Furthermore, perhaps through its influence on integrin-mediated responses, SIRP α regulates cellular adhesion and/or motility. For example, migration of monocytes or neutrophils is reduced by blocking the interaction of SIRP α with CD47 (21, 37), and the migration of fibroblasts derived from SIRP α mutant mice reveals defective cell spreading on fibronectin, although adhesion is enhanced (38). Furthermore, the process of giant cell formation by macrophage fusion involves phosphorylation of SIRP α , and blockade of the SIRP α -CD47 interaction is sufficient to inhibit fusion (24, 39). Transfection of SIRP α into the glioblastoma cell line U87MG similarly inhibits cell migration and spreading on fibronectin (40). Direct aggregation of Ba/F3 B cells to each other can be mediated by SIRP α -CD47 interactions (17), and neurite outgrowth and adhesion are augmented on surfaces coated with affinity-purified mouse SIRP α (14, 41).

Given the involvement of SIRP α in regulating mitogenesis, cell motility, and adhesion, its expression on tumor cells might be expected to alter their malignant properties. In support of this, SIRP α expression is reduced in blasts from both chronic and acute myeloid leukemias (42). By Northern blotting, SIRP α expression in hepatocellular carcinomas is inversely proportional to tumor size and/or metastasis (43, 44). Expression of transforming *v-src* in fibroblasts down-regulates SIRP α , whereas overexpression of SIRP α reduces anchorage-independent cell growth (45). Although SIRP α is not normally expressed on B cells, representational difference analysis of a Burkitt's lymphoma B-cell line has demonstrated up-regulation of SIRP α on immunoglobulin cross-linking (46). Recent reports have also shown reduced expression of SIRP α in breast cancer relative to paired normal breast tissues, as examined by Western blotting, although the cell types that expressed SIRP α were not identified (47). Finally, transfection of SIRP α into the glioblastoma cell line U87MG affects a number of tumor-related characteristics, including reduced focus formation, increased susceptibility to radiation-induced apoptosis, reduced cell migration and spreading, and reduced EGF-induced phosphatidylinositol 3'-kinase activation (40). Interestingly, in U87MG cells, SIRP α has no apparent effect on cell proliferation or mitogen-activated protein kinase (MAPK) activity as measured by extracellular signal-regulated kinase (ERK) 1, ERK2, and c-Jun NH₂-terminal kinase phosphorylation of *in vitro* substrates. This latter finding is similar to results in Chinese hamster ovary (CHO) cells, where expression of SIRP α does not affect phosphorylation of ERK1 and ERK2 in response to EGF (33). In contrast, SIRP α transfected into NIH3T3 cells inhibits EGF-stimulated phosphorylation of ERK1 and ERK2 (4), and SIRP α transfected into either NIH3T3 or Rat1 cells expressing insulin receptor augments insulin-stimulated phosphorylation of MAPK (32). Thus, the ultimate effects of SIRP α expression may depend on the cell in which it is expressed as well as the signaling complexes in question.

To address the role of SIRP α in the biology of human tumors, we generated a monoclonal antibody (mAb) against SIRPs and used this to survey a panel of cell lines derived from malignant brain tumors for SIRP expression. We found that five of nine cell lines expressed SIRP α , and all cell lines expressed CD47, the ligand for SIRP α 1. The SIRP α expressed on all astrocytoma cell lines bound to a soluble CD47 fusion protein. Additional studies of the astrocytoma cell lines SF126 and U373MG confirmed that the expressed SIRP is SIRP α 1,

that it can associate in these cells with the signaling adapter molecule SHP-2, and that its phosphorylation in cultured cells is CD47 dependent. Finally, we have shown by immunohistochemistry on 10 frozen sections that SIRPs and CD47 are expressed on primary brain tumor biopsies. To our knowledge, this is the first demonstration that SIRP α 1 is expressed on astrocytomas. The demonstration that SIRP α 1 can function in these cells may be important in the biological and malignant properties of astrocytomas.

MATERIALS AND METHODS

Cell Lines and Transfectants. Human astrocytoma cell lines were obtained courtesy of the Neurosurgery Tissue Bank at University of California San Francisco (UCSF). The cell lines A172 (48), SF126, SF210, SF268, SF295 (49), U87MG, U251, U343MG, and U373MG (50) have been described previously and were established by long-term culture of explants from brain tumor biopsies. CHO cells were obtained from the UCSF Cell Culture Facility. All cells were maintained at 37° in 5% CO₂ in RPMI 1640 (Mediatech, Herndon, VA) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin. Plasmids containing the genes for human SIRP α 1 and SIRP β 1 were kindly provided by Axel Ullrich (4). These genes were modified by addition of a sequence encoding the FLAG epitope (DYKDDDDK) at the 5' end of each expressed gene product. The genes for these FLAG-tagged proteins were then subcloned into the pMXneo (for SIRP α 1) or BSR α (for SIRP β 1) expression plasmids (obtained courtesy of Andrey Shaw; Washington University, St. Louis, MO), purified on Qiagen columns (Qiagen, Valencia, CA), transfected into CHO cells by using FuGENE (Roche, Indianapolis, IN), and selected with G418 at 1 mg/ml (Mediatech) or puromycin at 10 μ g/ml (Sigma, St. Louis, MO), respectively, to create the cell lines CHO-SIRP α and CHO-SIRP β . Expression was verified by the presence of a FLAG epitope detected with the anti-FLAG M2 antibody (Sigma).

Antibodies and Flow Cytometry. The B6H12 anti-CD47 antibody was obtained from PharMingen (San Diego, CA), and our 2D3 anti-CD47 antibody has been described previously (20). As described under "Results," the 2D3 mAb does not block binding of CD47 to SIRP α 1 but, instead, enhances it. The B-1 mouse mAb against SHP-2 and polyclonal goat antiserum (C-20) against a COOH-terminal peptide of SIRP α 1 were obtained from Santa Cruz Biotechnology Biotech (Santa Cruz, CA). A mAb against SIRP α was raised by immunizing BALB/c mice with CHO-SIRP α , constructed as described above. Mice were boosted four times, and spleens were harvested 3 days after the last boost and fused with SP2/0 cells using standard methods. Clones expressing antibodies with reactivity against 293T cells transfected with SIRP α 1, but not against untransfected cells, were subcloned and expanded. The resulting antibody, named 6.1, binds to both SIRP α 1 and SIRP β 1. Flow cytometry was performed by using a FACScan (BD Biosciences, San Jose, CA). Fluorescein-conjugated antimouse IgG antibodies were obtained from ICN (Aurora, OH). Phycoerythrin-conjugated antihuman IgG Fc-specific antibody was from Jackson ImmunoResearch Laboratories (West Grove, PA).

Binding of CD47 Fusion Protein to SIRP α . A fusion protein (CD47Fc) between the large extracellular loop of CD47 (which includes the ligand binding site for SIRP α) and the human IgG1 constant region has been described previously (20). To test for binding of CD47Fc to cells, 1 μ g of the fusion protein and 1 μ g of the enhancing antibody 2D3 were added to 10⁶ cells. Cells were incubated for 1 h on ice, washed extensively in PBS, and incubated with phycoerythrin-conjugated antibody against human Fc (Jackson ImmunoResearch Laboratories). This antibody showed no reactivity against mouse IgG and thus did not bind appreciably to cells coated with murine anti-CD47 antibodies. Binding was detected by flow cytometry as described above. Where indicated, the binding of CD47Fc to SIRP α was blocked by addition of the 6.1 anti-SIRP antibody at 5 μ g/ml.

Western Blotting. Cells were lysed with 1% digitonin, and immunoprecipitation was performed with the indicated mAb linked to protein G-Sepharose (Amersham, Piscataway, NJ), as described previously (51). Lysates were resolved by nonreducing 10% SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA) by semi-dry transfer. Membranes were blocked overnight at 4°C in Tris-buffered saline containing 0.05% Tween 20 (TBST) and 1% BSA, incubated with the appropriate primary antibody diluted

in the same solution for 1 h at room temperature, washed extensively in TBST, and then incubated with the appropriate second step antibody for 1 h at room temperature. Blots were again extensively washed in TBST, and bound antibody was visualized with SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL) and exposure to film (Hyperfilm, Amersham). Second-step antibodies used included horseradish peroxidase-conjugated sheep anti-mouse IgG antiserum (Amersham) and horseradish peroxidase-conjugated swine anti-goat IgG antiserum (Caltag, Burlingame, CA).

Deglycosylation of Proteins. After immunoprecipitation with mAb 6.1, lysates were denatured in 0.5% SDS, and phenylmethanesulfonyl fluoride was added to 1 mM. Deglycosylation was performed with PNGaseF (New England Biolabs, Beverly, MA) overnight at 37°C per the manufacturer's directions, after which samples were subjected to SDS-PAGE and Western blotting as described above, using the C-20 goat antiserum against the SIRP α cytoplasmic domain (Santa Cruz Biotechnology Biotech).

Northern Blotting. Total RNA was isolated from cells by using TRIzol (Life Technologies, Inc., Gaithersburg, MD), and 50 μ g were enriched for polyadenylated RNA by using Oligotex beads (Qiagen). RNA was separated on 1% agarose formaldehyde gels and transferred to Hybond-N+ (Amersham) by capillary transfer in 20 \times SSC. Membranes were cross-linked by baking at 80°C for 2 h, prehybridized for 1 h at 42°C with UltraHyb (Ambion, Austin, TX), and hybridized overnight to a DNA probe from the 3' end of the SIRP α gene labeled with ³²P (Rediprime II; Amersham). Membranes were washed at medium stringency and exposed to film (BioMax MS; Kodak, Rochester, NY). To standardize loading, after probing for SIRP α 1, the blot was stripped by incubating membranes for 30 min in 0.1 \times SSC/1% SDS at 80°C. The membranes were then reprobed with a ³²P-labeled probe for β -actin.

Reverse Transcription, PCR, and Sequencing. First-strand cDNA was synthesized from RNA, isolated as described above, by using either oligo(dT)₁₂₋₁₈ (Roche) or a primer specific to the 3'-untranslated region of SIRP α (5'-CGGGGAGGAGTCGAGGGTCTTCAAAAC-3') together with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's directions. After first-strand synthesis was complete, RNA was degraded with RNaseH (Roche), and 1 μ l was used for subsequent PCR amplification using Taq polymerase (Roche). To clone the bulk of the coding region of SIRP α , the following primer pair was used: 5'-GGCGGGTGAGGAGGCTGCAGGTGAT-3' (predicted to hybridize upstream from the V region) and 5'-GCGGGCTGCGGGCTGGTCTGAATG-3' (predicted to hybridize near the second ITIM motif). To clone the cytoplasmic tail and part of the 3'-untranslated region, the following primer pair was used: 5'-GCCGAGAAGAATGCCAGAGAAATAACACA-3' (predicted to hybridize between the transmembrane domain and the first ITIM) and 5'-CGGGGAGCGAGTCGAGGGTCTTCAAAAC-3' (predicted to hybridize 320 bp downstream of the stop codon). PCR fragments were resolved on 1.2% agarose gels, purified with the QIAquick gel extraction kit (Qiagen), and cloned using the TOPO PCR cloning system (Invitrogen). All sequencing and oligonucleotide synthesis was performed at the Biomolecular Resource Center at UCSF.

Pervanadate Stimulation and SHP-2 Association. Tyrosine phosphorylation of cell proteins was nonspecifically enhanced by incubation in 10 mM sodium orthovanadate and 0.6% hydrogen peroxide (pervanadate) for 5 min as described previously (51). After incubation, cells were rapidly pelleted and lysed in digitonin as described above. Immunoprecipitation with the B-1 anti-SHP-2 antibody linked to protein G-Sepharose and Western blotting with the 6.1 anti-SIRP α antibody were performed as described above. Blots were subsequently stripped under mild conditions (ReBlot Plus; Chemicon, Temecula, CA) and reprobed with anti-SHP-2 antibody (Transduction Laboratories, Lexington, KY).

Cell Stimulation by Aggregation. To stimulate cells by aggregation, astrocytoma cell lines were harvested, washed twice in PBS, resuspended in serum-free RPMI 1640 supplemented with 1% low endotoxin Cohn analogue BSA (Sigma), and added to standard tissue culture plates (Corning, NY) at 5 \times 10⁶ cells/plate. When indicated, plates were coated with vitronectin by incubating plates overnight at 4°C with human vitronectin (Sigma) at 10 μ g/ml. Appropriate blocking antibodies were added at 5 μ g/ml. After overnight incubation at 37°C in 5% CO₂, digitonin cell lysates were prepared and subjected to immunoprecipitation, resolved on SDS-PAGE, transferred to polyvinylidene difluoride, and analyzed for phosphotyrosine by using the mAb 4G10 as described above. To verify the level of SIRP α , blots were reprobed with biotinylated 6.1 anti-SIRP antibody and alkaline phosphatase-conjugated

streptavidin (Pierce), and bound complexes were detected using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate (Roche) per the manufacturer's directions.

Immunohistochemistry. Multiple sections from frozen specimens of brain tumor biopsies were obtained from the Neurosurgery Tissue Bank at UCSF through a protocol approved by the Human Studies Committee. Sections were stored desiccated at -20°C until ready for use. One section was stained with H&E and evaluated by one of us (E. J. H.) to verify the diagnosis of grade 4 astrocytoma and to quantify the percentage of tumor in each sample. To perform immunohistochemistry with anti-SIRP (mAb 6.1) or anti-CD47 (mAb B6H12), slides were fixed for 5 min in acetone, washed twice in PBS, and incubated with serum-free blocking solution (DAKO, Carpinteria, CA) for 1 h at room temperature. Slides were washed twice in PBS and incubated for 1 h at room temperature with the primary antibody diluted to 1 μ g/ml in PBS with 1% BSA. Bound antibodies were detected by using the LSAB2 avidin-biotin complex system (DAKO) and 3,3'-diaminobenzidine per the manufacturer's directions. After chromogen development, cells were counterstained for 5 min with hematoxylin (Sigma), dehydrated in xylene, and mounted with Permount (Fisher) per standard protocols. Slides were subsequently read in a blinded fashion by E. J. H. for binding of antibody to SIRP or to CD47. The method of scoring is presented in "Results."

RESULTS

SIRP α and CD47 Are Expressed on Astrocytoma Cell Lines.

To assess the expression of SIRPs on the cell surface, we generated a mouse mAb against SIRPs by immunizing BALB/c mice with CHO cells transfected with a gene encoding SIRP α 1. One antibody, 6.1, reacts with SIRP α 1 expressed on either CHO cells or 293T cells, but not untransfected cells. This antibody cross-reacts with SIRP β 1, reflecting the extensive homology between the extracellular domain of these two genes (4). To test for expression of SIRPs on astrocytoma cell lines, the 6.1 antibody was used in fluorescence analysis of a panel of nine cell lines obtained from the Neurosurgery Tissue Bank at UCSF. As shown in Fig. 1, the 6.1 antibody reacted with five of the cell lines tested (SF126, SF210, SF268, U251, and U373MG). Ex-

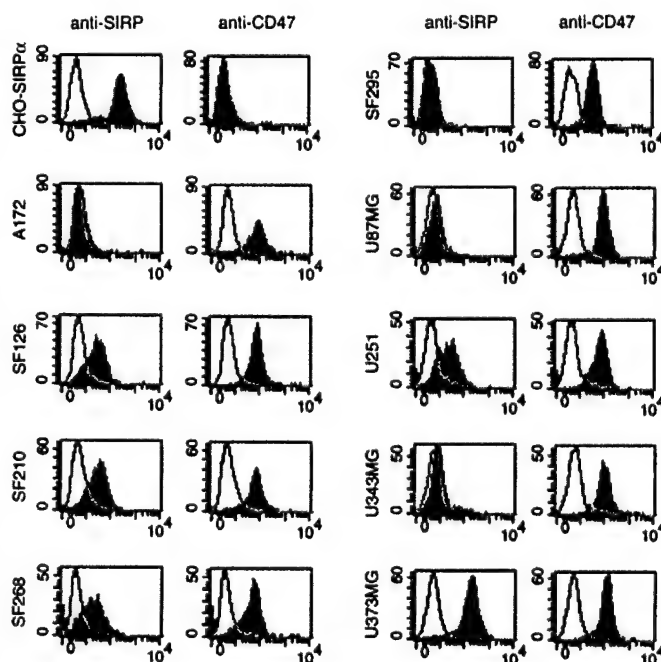


Fig. 1. Fluorescence-activated cell-sorting analysis of astrocytoma cell lines. Anti-signal regulatory protein or anti-CD47 antibodies (filled histograms) or an isotype-matched control antibody (open histograms) was used in flow cytometry on the indicated cell lines as described in "Materials and Methods." Binding was detected with a fluorescein-conjugated secondary antibody.

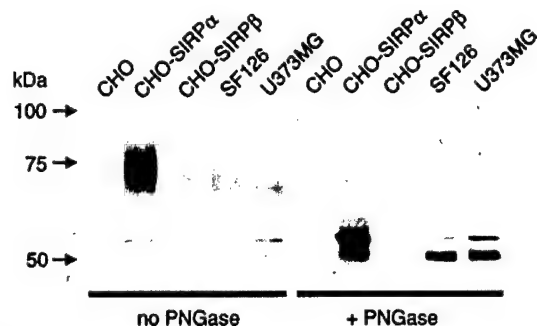


Fig. 2. Immunoprecipitation and Western blotting of astrocytoma cell lines detects signal regulatory protein (SIRP) α . Digitonin lysates were prepared from the indicated cell lines and subjected to immunoprecipitation with anti-SIRP α antibody loaded on protein G-Sepharose beads. Half of the immunoprecipitated protein was subjected to enzymatic deglycosylation with PNGaseF as described in "Materials and Methods." Lysates were then resolved under nonreducing conditions on 10% SDS-PAGE and subjected to Western blotting with an antiserum specific for SIRP α . Positions of molecular weight markers are indicated on the border (in thousands).

pression of SIRPs on two other cell lines (U87MG and U343MG) was intermittently detectable above background, whereas SIRP expression on the remaining two cell lines (A172 and SF295) was consistently negative. No reaction with any cell line was observed with an isotype-matched control antibody. Of the cell lines tested, U373MG expressed the highest level of SIRPs. We also tested all cell lines for expression of CD47, a known ligand for SIRP α , by using the antibody B6H12. All cell lines expressed high levels of CD47 (Fig. 1).

The SIRP Expressed on Astrocytoma Cell Lines Is SIRP α and Is Underglycosylated. The above-mentioned studies demonstrated that SIRPs are frequently present on astrocytoma cell lines. However, the 6.1 anti-SIRP antibody does not distinguish between SIRP α and SIRP β . To formally test for the expression of SIRP α , two astrocytoma cell lines demonstrating the highest reactivity to the 6.1 mAb (SF126 and U373MG) were subjected to immunoprecipitation with mAb 6.1 followed by Western blotting with a commercial antiserum against a peptide in the cytoplasmic tail of SIRP α 1. SIRP β does not contain a homologous region and thus does not react with this antiserum. As controls, lysates prepared from CHO cells, CHO cells transfected with the SIRP α 1 gene (CHO-SIRP α 1), and CHO cells transfected with the SIRP β 1 gene (CHO-SIRP β 1) were also analyzed in parallel. As shown in the first five lanes (*no PNGase*) of Fig. 2, the 6.1 antibody immunoprecipitated a protein that is recognized by the SIRP α -specific antiserum in the CHO-SIRP α 1, SF126, and U373MG cell lines but not the untransfected CHO cells or the CHO-SIRP β 1 line. This band migrates at M_r 70,000–90,000 in CHO-SIRP α 1 but at M_r 65,000–75,000 in the astrocytoma cell lines.

Additional studies demonstrated that the difference in molecular weight between SIRP α from CHO-SIRP α 1 and SIRP α astrocytoma cell lines reflects differences in glycosylation. For these studies, cell lysates were immunoprecipitated as described above and then subjected to deglycosylation with PNGaseF (Fig. 2, *+ PNGase*). After treatment, SIRP α from both CHO-SIRP α and the astrocytoma cell lines bands migrated as a doublet at M_r 53,000–57,000. The smaller size is the predicted molecular weight for human SIRP α (4), and the larger band likely represents incomplete deglycosylation. Thus, the discrepancy in molecular weight between SIRP α expressed on transfected CHO cells *versus* SIRP α on astrocytomas reflects differences in glycosylation. A similar reduced glycosylation of SIRP α expressed in the rat brain has been noted by others (23).

Astrocytoma Cell Lines Express Transcripts for SIRP α 1 as Assessed by PCR. The initial characterization of SIRP α by Khari-tonenkov *et al.* (4) revealed a family of proteins that differ primarily in the first immunoglobulin (V) domain (3), as well as several forms

due to alternative splicing (11, 52). To establish the identity of the SIRP α protein expressed on astrocytoma cell lines, PCR primers were designed based on published sequences of SIRP α receptors and used to clone the expressed gene from both the SF126 and U373MG cell lines. These primer sequences are common to each of the three best-characterized subtypes of SIRP α (SIRP α 1, SIRP α 2, and SIRP α 3). The predominant sequences obtained from the SF126 and U373MG cell lines were identical to the published SIRP α 1 sequence in the extracellular domain (4), and are represented by the sequence U373-1 in Fig. 3. Also shown in Fig. 3 are the sequences of three other clones obtained from the U373MG cell line, aligned to the published sequences of SIRP α 1 and SIRP α 2. A single clone, obtained from the U373MG cell line, is identical to the SIRP α 2 sequence in the V domain (sequence U373-4 in Fig. 3). We have also detected transcripts that correspond to the previously described splice variant of SIRP α 1 missing the second and third immunoglobulin domains (shown as U373-2), and we have also detected transcripts containing previously undescribed splice variants (shown as U373-3 and U373-4). These latter sequences are similar to U373-2 in that the extracellular sequences contain only the V domain, but they differ in that they are missing the transmembrane domain and variable amounts of the intracellular tail. Thus, PCR amplification of SIRP transcripts from astrocytoma cell lines detected primarily SIRP α 1, including full-length and alternatively spliced transcripts. One transcript from U373MG cells, however, was SIRP α 2.

Astrocytoma Cell Lines Express Primarily Transcripts for Complete (Three Immunoglobulin Domain) SIRP α by Northern Blotting. Having demonstrated that SIRP α 1 is expressed in both SF126 and U373MG astrocytoma cells, we next examined expression of SIRP α transcripts on all cell lines by Northern blotting. We used a probe derived from the 3'-untranslated region of the SIRP α 1 gene, which is retained in both the full-length and alternatively spliced forms but is not present in SIRP β . The results, shown in Fig. 4, demonstrate bands at 4.2 and 2.7 kb, which reflect the expected sizes of the alternatively polyadenylated forms of SIRP α described previously (11, 52). The mature mRNA encoding the single-immunoglobulin domain splice variant would be predicted to be approximately 650 bp shorter than the full-length mRNA. Despite overexposure of several Northern blots, no bands corresponding to this predicted splice variant were consistently detected (data not shown). Therefore, although the single immunoglobulin domain splice variant was detectable by PCR, it is likely a low abundance transcript compared with the full-length mRNA.

Surprisingly, all but one of the astrocytoma cell lines expressed SIRP α transcripts as assessed by Northern blotting, despite quite variable cell surface expression as determined by flow cytometry (see Fig. 1). There was no clear correlation between mRNA abundance and cell surface expression, and one cell line with abundant mRNA for SIRP α expressed no detectable SIRP α on its cell surface (SF295), whereas cell lines with modest levels of mRNA for SIRP α expressed high levels of SIRP α on the cell surface (SF210 and SF268). Nonetheless, U373MG cells had both the most abundant transcripts (as assayed by Northern blotting) and the highest levels of SIRP expression (as assessed by flow cytometry). These findings suggest that SIRP α protein expression is regulated in part at the posttranscriptional level. Alternatively, posttranslational modification of SIRP α 1 may produce isoforms that are not recognized by our antibody.

SIRP α 1 on Astrocytoma Cell Lines Binds to CD47. To begin characterizing the functional significance of SIRP α 1 expression on astrocytoma cell lines, we next examined the binding of SIRP α 1 on astrocytoma cell lines to its known ligand, CD47. Prior studies with rodent and human cells have shown that SIRP α fusion proteins bind CD47 (10, 16, 18, 42). Similarly, a CD47 fusion protein (CD47Fc) is

		IG1 (V)											
SIRPa2	GGCGGGTGAG	GAGGAGCTGC	AGGTGATTCA	GGCTGACAAG	TCGGTGTGG	TGGCAGCTGG	AGAGACAGCC	ACTCTGGCGT	GCACTGGGAC	CTCTCTGATC			
SIRPa1	GGCGGGTGAG	GAGGAGCTGC	AGGTGATTCA	GGCTGACAAG	TCGGTGTGG	TGGCAGCTGG	AGAGACAGCC	ACTCTGGCGT	GCACTGGGAC	CTCTCTGATC			
U373-1	GGCGGGTGAG	GAGGAGCTGC	AGGTGATTCA	GGCTGACAAG	TCGGTGTGG	TGGCAGCTGG	AGAGACAGCC	ACTCTGGCGT	GCACTGGGAC	CTCTCTGATC			
U373-2	GGCGGGTGAG	GAGGAGCTGC	AGGTGATTCA	GGCTGACAAG	TCGGTGTGG	TGGCAGCTGG	AGAGACAGCC	ACTCTGGCGT	GCACTGGGAC	CTCTCTGATC			
U373-3	GGCGGGTGAG	GAGGAGCTGC	AGGTGATTCA	GGCTGACAAG	TCGGTGTGG	TGGCAGCTGG	AGAGACAGCC	ACTCTGGCGT	GCACTGGGAC	CTCTCTGATC			
U373-4	GGCGGGTGAG	GAGGAGCTGC	AGGTGATTCA	GGCTGACAAG	TCGGTGTGG	TGGCAGCTGG	AGAGACAGCC	ACTCTGGCGT	GCACTGGGAC	CTCTCTGATC			
SIRPa2	CCCTGTGGGG	CCATCCAGTG	GTTTCAGAGG	GCTGGACCAG	CCCGGGGAATT	AATCTACAAT	CAAAAAGAG	GCCACTTCCC	CCGGGTAAAC	ACTGTTTCAG			
SIRPa1	CCCTGTGGGG	CCATCCAGTG	GTTTCAGAGG	GCTGGACCAG	CCCGGGGAATT	AATCTACAAT	CAAAAAGAG	GCCACTTCCC	CCGGGTAAAC	ACTGTTTCAG			
U373-1	CCCTGTGGGG	CCATCCAGTG	GTTTCAGAGG	GCTGGACCAG	CCCGGGGAATT	AATCTACAAT	CAAAAAGAG	GCCACTTCCC	CCGGGTAAAC	ACTGTTTCAG			
U373-2	CCCTGTGGGG	CCATCCAGTG	GTTTCAGAGG	GCTGGACCAG	CCCGGGGAATT	AATCTACAAT	CAAAAAGAG	GCCACTTCCC	CCGGGTAAAC	ACTGTTTCAG			
U373-3	CCCTGTGGGG	CCATCCAGTG	GTTTCAGAGG	GCTGGACCAG	CCCGGGGAATT	AATCTACAAT	CAAAAAGAG	GCCACTTCCC	CCGGGTAAAC	ACTGTTTCAG			
U373-4	CCCTGTGGGG	CCATCCAGTG	GTTTCAGAGG	GCTGGACCAG	CCCGGGGAATT	AATCTACAAT	CAAAAAGAG	GCCACTTCCC	CCGGGTAAAC	ACTGTTTCAG			
SIRPa2	ACCTCACAAA	GAGAAACAAC	ATGGACTTTT	CCATCCGCAT	CGGTAACATC	ACCCACGACG	ATGCCGGCAC	CTACTACTGT	GTGAAGTTCC	GGAAAGGGAG			
SIRPa1	AGTCCACAAA	GAGAGAAAAC	ATGGACTTTT	CCATCCGCAT	CAGTAACATC	ACCCACGACG	ATGCCGGCAC	CTACTACTGT	GTGAAGTTCC	GGAAAGGGAG			
U373-1	AGTCCACAAA	GAGAGAAAAC	ATGGACTTTT	CCATCCGCAT	CAGTAACATC	ACCCACGACG	ATGCCGGCAC	CTACTACTGT	GTGAAGTTCC	GGAAAGGGAG			
U373-2	AGTCCACAAA	GAGAGAAAAC	ATGGACTTTT	CCATCCGCAT	CAGTAACATC	ACCCACGACG	ATGCCGGCAC	CTACTACTGT	GTGAAGTTCC	GGAAAGGGAG			
U373-3	AGTCCACAAA	GAGAGAAAAC	ATGGACTTTT	CCATCCGCAT	CAGTAACATC	ACCCACGACG	ATGCCGGCAC	CTACTACTGT	GTGAAGTTCC	GGAAAGGGAG			
U373-4	AGTCCACAAA	GAGAGAAAAC	ATGGACTTTT	CCATCCGCAT	CAGTAACATC	ACCCACGACG	ATGCCGGCAC	CTACTACTGT	GTGAAGTTCC	GGAAAGGGAG			
SIRPa2	CCCGGATGAC	GTGGAGTTTA	AGTCTGAGG	AGGCACTGAG	CTGTCTGTGC	GTGCTGAGG	CTGTGCCCCC	GTGGTATCGG	CCCTCTGCGG	GAGGCCACAA			
SIRPa1	CCCGGATGAC	GTGGAGTTTA	AGTCTGAGG	AGGCACTGAG	CTGTCTGTGC	GTGCTGAGG	CTGTGCCCCC	GTGGTATCGG	CCCTCTGCGG	GAGGCCACAA			
U373-1	CCCGGATGAC	GTGGAGTTTA	AGTCTGAGG	AGGCACTGAG	CTGTCTGTGC	GTGCTGAGG	CTGTGCCCCC	GTGGTATCGG	CCCTCTGCGG	GAGGCCACAA			
U373-2	CCCGGATGAC	GTGGAGTTTA	AGTCTGAGG	AGGCACTGAG	CTGTCTGTGC	GTGCTGAGG	CTGTGCCCCC	GTGGTATCGG	CCCTCTGCGG	GAGGCCACAA			
U373-3	CCCGGATGAC	GTGGAGTTTA	AGTCTGAGG	AGGCACTGAG	CTGTCTGTGC	GTGCTGAGG	CTGTGCCCCC	GTGGTATCGG	CCCTCTGCGG	GAGGCCACAA			
U373-4	CCCGGATGAC	GTGGAGTTTA	AGTCTGAGG	AGGCACTGAG	CTGTCTGTGC	GTGCTGAGG	CTGTGCCCCC	GTGGTATCGG	CCCTCTGCGG	GAGGCCACAA			
IG#2													
SIRPa2	CCTCAGCACA	CAGTGAGCTT	CACCTGCGAG	TCCCACGGGT	TCTCACCAG	AGACATCAAC	CTGAAATGGT	TCAAAAATGG	GAATGAGCTC	TCAGACTTCC			
SIRPa1	CCTCAGCACA	CAGTGAGCTT	CACCTGCGAG	TCCCACGGGT	TCTCACCAG	AGACATCAAC	CTGAAATGGT	TCAAAAATGG	GAATGAGCTC	TCAGACTTCC			
U373-1	CCTCAGCACA	CAGTGAGCTT	CACCTGCGAG	TCCCACGGGT	TCTCACCAG	AGACATCAAC	CTGAAATGGT	TCAAAAATGG	GAATGAGCTC	TCAGACTTCC			
U373-2			
U373-3			
U373-4			
SIRPa2	AGACCAACGT	GGACCCCGTA	GGAGAGAGCG	TGTCTCTACG	CATCCACAGC	ACAGCCAAGG	TGGTGCTGAC	CCGCGAGGAC	GTTCACCTCTC	AAGTCATCTG			
SIRPa1	AGACCAACGT	GGACCCCGTA	GGAGAGAGCG	TGTCTCTACG	CATCCACAGC	ACAGCCAAGG	TGGTGCTGAC	CCGCGAGGAC	GTTCACCTCTC	AAGTCATCTG			
U373-1	AGACCAACGT	GGACCCCGTA	GGAGAGAGCG	TGTCTCTACG	CATCCACAGC	ACAGCCAAGG	TGGTGCTGAC	CCGCGAGGAC	GTTCACCTCTC	AAGTCATCTG			
U373-2			
U373-3			
U373-4			
SIRPa2	CGAGGTGGCC	CAGCTCAACT	TGCAGGGGGA	CCCTCTTCTG	GGGACTGCGA	ACTTGTCTGA	GACCATCCGA	GTTCACCCCA	CCTTGGAGGT	TACTCAACAG			
SIRPa1	CGAGGTGGCC	CAGCTCAACT	TGCAGGGGGA	CCCTCTTCTG	GGGACTGCGA	ACTTGTCTGA	GACCATCCGA	GTTCACCCCA	CCTTGGAGGT	TACTCAACAG			
U373-1	CGAGGTGGCC	CAGCTCAACT	TGCAGGGGGA	CCCTCTTCTG	GGGACTGCGA	ACTTGTCTGA	GACCATCCGA	GTTCACCCCA	CCTTGGAGGT	TACTCAACAG			
U373-2			
U373-3			
U373-4			
IG#3													
SIRPa2	CCCGTGAGGG	CAGAGAACCA	GGTGAATGTC	ACCTGCCAGG	TGAGGAAGTT	CTACCCCCAG	AGACTACAGC	TGACCTGGTT	GGAGAATGGA	AACGTGTCCC			
SIRPa1	CCCGTGAGGG	CAGAGAACCA	GGTGAATGTC	ACCTGCCAGG	TGAGGAAGTT	CTACCCCCAG	AGACTACAGC	TGACCTGGTT	GGAGAATGGA	AACGTGTCCC			
U373-1	CCCGTGAGGG	CAGAGAACCA	GGTGAATGTC	ACCTGCCAGG	TGAGGAAGTT	CTACCCCCAG	AGACTACAGC	TGACCTGGTT	GGAGAATGGA	AACGTGTCCC			
U373-2			
U373-3			
U373-4			
SIRPa2	GGACAGAAAC	GGCCTCAACC	GTTACAGAGA	ACAAGGATGG	TACCTACAA	TGGATGAGCT	GGCTCTGGT	GAATGTATCT	GGCCACAGGG	ATGATGTGAA			
SIRPa1	GGACAGAAAC	GGCCTCAACC	GTTACAGAGA	ACAAGGATGG	TACCTACAA	TGGATGAGCT	GGCTCTGGT	GAATGTATCT	GGCCACAGGG	ATGATGTGAA			
U373-1	GGACAGAAAC	GGCCTCAACC	GTTACAGAGA	ACAAGGATGG	TACCTACAA	TGGATGAGCT	GGCTCTGGT	GAATGTATCT	GGCCACAGGG	ATGATGTGAA			
U373-2			
U373-3			
U373-4			
SIRPa2	GCTCACTGCG	CAGGTGGAGC	ATGACGGGCA	GCCAGGGGTC	AGCAAAAGCC	ATGACCTGAA	GGCTCTGAGC	CACCCGAAAG	AGCAGGGGTC	AAATACGGCC			
SIRPa1	GCTCACTGCG	CAGGTGGAGC	ATGACGGGCA	GCCAGGGGTC	AGCAAAAGCC	ATGACCTGAA	GGCTCTGAGC	CACCCGAAAG	AGCAGGGGTC	AAATACGGCC			
U373-1	GCTCACTGCG	CAGGTGGAGC	ATGACGGGCA	GCCAGGGGTC	AGCAAAAGCC	ATGACCTGAA	GGCTCTGAGC	CACCCGAAAG	AGCAGGGGTC	AAATACGGCC			
U373-2			
U373-3			
U373-4			
Transmembrane													
SIRPa2	GCTGAGAAC	CTGGATCTAA	TGAACGGGAC	ATCTATATTG	TGGTGGGTGT	GCTGTGCACC	TGCTGGTGGT	CCCTACTGAT	GGGGGCCCTC	TACCTGTGTC			
SIRPa1	GCTGAGAAC	CTGGATCTAA	TGAACGGGAC	ATCTATATTG	TGGTGGGTGT	GCTGTGCACC	TGCTGGTGGT	CCCTACTGAT	GGGGGCCCTC	TACCTGTGTC			
U373-1	GCTGAGAAC	CTGGATCTAA	TGAACGGGAC	ATCTATATTG	TGGTGGGTGT	GCTGTGCACC	TGCTGGTGGT	CCCTACTGAT	GGGGGCCCTC	TACCTGTGTC			
U373-2	...AGAACA	CTGGATCTAA	TGAACGGGAC	ATCTATATTG	TGGTGGGTGT	GCTGTGCACC	TGCTGGTGGT	CCCTACTGAT	GGGGGCCCTC	TACCTGTGTC			
U373-3			
U373-4			
SIRPa2	GAATCAGACA	GAAGAAAGCC	CAGGGCTCCA	CTTCTCTAC	AAGGTTGCAT	GAGCCCGAGA	AGAATGCCAG	AGAAATAACA	CAGGACACAA	ATGATATCAC			
SIRPa1	GAATCAGACA	GAAGAAAGCC	CAGGGCTCCA	CTTCTCTAC	AAGGTTGCAT	GAGCCCGAGA	AGAATGCCAG	AGAAATAACA	CAGGACACAA	ATGATATCAC			
U373-1	GAATCAGACA	GAAGAAAGCC	CAGGGCTCCA	CTTCTCTAC	AAGGTTGCAT	GAGCCCGAGA	AGAATGCCAG	AGAAATAACA	CAGGACACAA	ATGATATCAC			
U373-2	GAATCAGACA	GAAGAAAGCC	CAGGGCTCCA	CTTCTCTAC	AAGGTTGCAT	GAGCCCGAGA	AGAATGCCAG	AGAAATAACA	CAGGACACAA	ATGATATCAC			
U373-3CC	CAGGGCTCCA	CTTCTCTAC	AAGGTTGCAT	GAGCCCGAGA	AGAATGCCAG	AGAAATAACA	CAGGACACAA	ATGATATCAC	ATGATATCAC			
U373-4			
SIRPa2	ATATGCAGAC	CTGAACCTGC	CCAAGGGGAA	GAAGCTTGCT	CCCGAGGCTG	CGGAGGCCAA	CAACCCACAG	GAGTATGCCA	GCATTTCAGC	CAGCC			
SIRPa1	ATATGCAGAC	CTGAACCTGC	CCAAGGGGAA	GAAGCTTGCT	CCCGAGGCTG	CGGAGGCCAA	CAACCCACAG	GAGTATGCCA	GCATTTCAGC	CAGCC			
U373-1	ATATGCAGAC	CTGAACCTGC	CCAAGGGGAA	GAAGCTTGCT	CCCGAGGCTG	CGGAGGCCAA	CAACCCACAG	GAGTATGCCA	GCATTTCAGC	CAGCC			
U373-2	ATATGCAGAC	CTGAACCTGC	CCAAGGGGAA	GAAGCTTGCT	CCCGAGGCTG	CGGAGGCCAA	CAACCCACAG	GAGTATGCCA	GCATTTCAGC	CAGCC			
U373-3	ATATGCAGAC	CTGAACCTGC	CCAAGGGGAA	GAAGCTTGCT	CCCGAGGCTG	CGGAGGCCAA	CAACCCACAG	GAGTATGCCA	GCATTTCAGC	CAGCC			
U373-4	ATATGCAGAC	CTGAACCTGC	CCAAGGGGAA	GAAGCTTGCT	CCCGAGGCTG	CGGAGGCCAA	CAACCCACAG	GAGTATGCCA	GCATTTCAGC	CAGCC			

Fig. 3. Sequence of signal regulatory protein α genes expressed in U373MG cells. Signal regulatory protein α was cloned from U373MG as described in "Materials and Methods" using PCR. Sequences corresponding to the three immunoglobulin domains (IG#1, IG#2, and IG#3) are shaded, sequences corresponding to the transmembrane domain are overlined, and predicted ITIM motifs are boxed.

capable of binding to SIRP α expressed on L cells (20). In these studies, we examined binding by the CD47Fc fusion protein in the presence of an antibody to CD47 (2D3) that does not block binding of CD47Fc to SIRP α 1 but, instead, enhances it. Enhanced binding is not due simply to multimerization of CD47 because enhancement is also seen with Fab fragments of 2D3 antibodies.⁶ In control studies, CD47Fc bound readily to CHO-SIRP α but not to untransfected CHO cells or to CHO-SIRP β 1 (Fig. 5A, top row, left three panels).

By using the combination of CD47Fc and the 2D3 enhancing

antibody, we found that all astrocytoma cell lines that expressed SIRP α bound to CD47Fc. Binding to most astrocytoma cells was only detectable in the presence of the enhancing antibody 2D3, with the exception of the U373MG cell line, which expressed the highest level of SIRP α (see Fig. 1; data not shown). In general, the level of binding by CD47Fc correlated with intensity of binding by the anti-SIRP antibody 6.1 (Figs. 1 and 5A). Binding of CD47Fc could be blocked by excess 6.1 antibody in the SF126 and U373MG cell lines (Fig. 5B), providing additional evidence that this interaction is specific. We conclude that SIRP α expressed on these astrocytoma cell lines is capable of binding CD47, despite underglycosylation compared with

⁶ E. J. Brown, unpublished results.

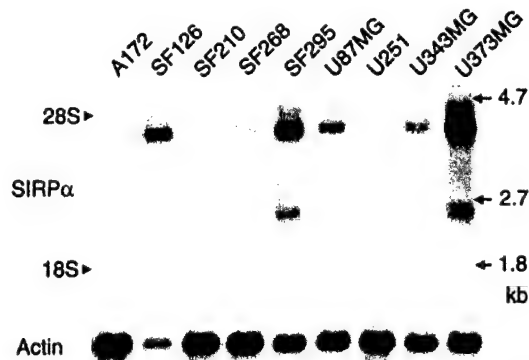


Fig. 4. Northern blot of astrocytoma cell lines. Polyadenylated-enriched RNA from the indicated cell lines was separated on a 1% denaturing formaldehyde gel, transferred to a nylon membrane, and hybridized to a signal regulatory protein α -specific probe (top panel) as described in "Materials and Methods." Positions of molecular weight marker are indicated on the right, and positions of the 28S and 18S rRNA bands as detected by methylene blue staining are indicated on the left. After detection of signal regulatory protein α -specific bands, the membrane was stripped and rehybridized to a probe for β -actin (bottom panel).

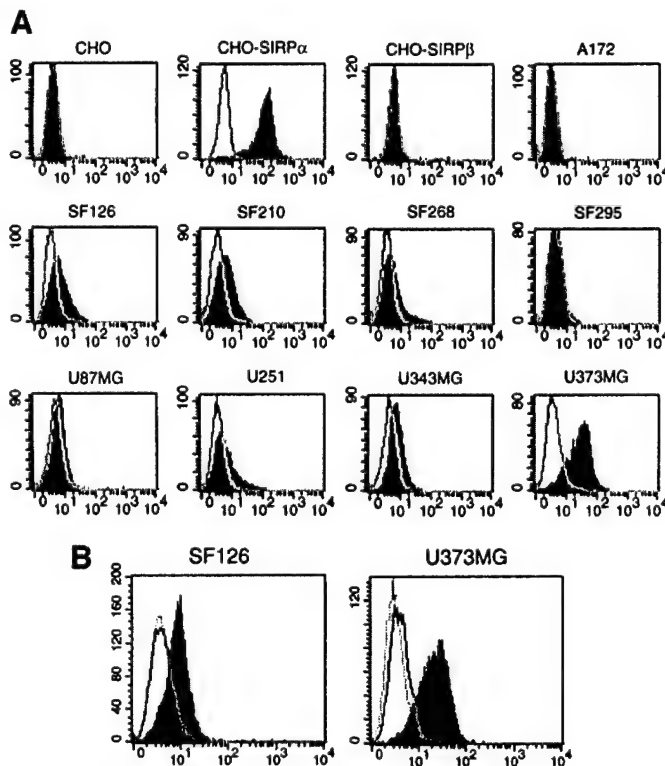


Fig. 5. Binding of CD47Fc fusion protein assayed by fluorescence-activated cell sorting (FACS) analysis. A, indicated cell lines were incubated with the 2D3 enhancing antibody alone (open histograms) or the CD47Fc fusion protein with 2D3 (filled histograms) as described in "Materials and Methods." Binding was detected with a phycoerythrin-conjugated secondary antibody specific to human Fc and FACS analysis. B, binding of CD47Fc fusion protein to SF126 (left panel) or U373MG cells (right panel) was performed as described above (open and filled histograms), but binding was also blocked with 5 μ g/ml anti-signal regulatory protein α antibody (dotted histograms). Subsequent binding was detected with a phycoerythrin-conjugated secondary antibody specific to human Fc and FACS analysis as described above.

SIRP α expressed on CHO cells. These results also indicate that our mAb to SIRP α 1 detects all forms of SIRP α 1 that are capable of binding CD47.

Phosphorylation of SIRP α on Astrocytoma Cell Lines Leads to Its Association with SHP-2. Phosphorylation of the ITIM motifs in the SIRP α cytoplasmic domain mediates the recruitment of the tyrosine phosphatase SHP-2 (3, 4). To establish whether this association

also occurs in astrocytoma cell lines that express SIRP α 1, two lines (SF126 and U373MG) were incubated in pervanadate to nonspecifically block phosphatase activity and thus enhance protein tyrosine phosphorylation. Cell lysates were then subjected to immunoprecipitation with anti-SHP-2 antibody and then assayed by Western blotting with the 6.1 anti-SIRP antibody. As shown in Fig. 6, SIRP α is coimmunoprecipitated with SHP-2 when, and only when, cells are stimulated with sodium orthovanadate. Thus, on phosphorylation, SIRP α is capable of recruiting SHP-2 in astrocytoma cells.

SIRP α Is Phosphorylated in Astrocytoma Cell Lines in Response to Interaction with CD47. To further establish that SIRP α can be activated in the astrocytoma cell lines, we examined the effect of cell adhesion on the phosphorylation of SIRP α . For these studies, astrocytoma cell lines were grown under serum-free conditions to prevent serum stimulation of SIRP α phosphorylation, after which SIRP α was immunoprecipitated from cell lysates and examined for phosphotyrosine by Western blotting. In hematopoietic cells and transfected cell lines, SIRP α is phosphorylated on adhesion to various extracellular matrix proteins, including vitronectin, laminin, and fibronectin (3, 26, 27). Therefore, we examined cells grown in either the absence or presence of extracellular matrix. By observation, under serum-free conditions, astrocytoma cell lines incubated in uncoated plates did not adhere well to the plates but rather tended to aggregate with each other, whereas cell lines incubated on vitronectin-coated plates adhered with normal morphology (data not shown). Because all of the cell lines express CD47, the ligand for SIRP α , cell aggregation could potentially be due to an interaction between CD47 and SIRP α . However, this was evidently not the only factor mediating astrocytoma aggregation because cell lines without detectable SIRP α expression also adhered to each other, and addition of blocking antibodies to either CD47 or SIRP α did not block this aggregation (data not shown).

Having observed the morphological effects of cell adhesion either to an extracellular matrix protein or to each other, we studied the effects of this adhesion on SIRP α phosphorylation by anti-phosphotyrosine Western blotting. SF126 or U373MG cell suspensions were prepared in serum-free media and incubated either on uncoated plates or vitronectin-coated plates, with either an excess of an anti-CD47 antibody that blocked its interaction with SIRP α (B6H12), a non-blocking anti-CD47 antibody (2D3), or an isotype control antibody. Cell viability or the extent of aggregation was unaffected by the addition of either blocking antibody (data not shown). SIRP α was heavily phosphorylated on cell aggregation, and this phosphorylation was inhibited when the SIRP α -CD47 interaction was blocked by B6H12 (Fig. 7). Phosphorylation of SIRP α was similarly blocked by

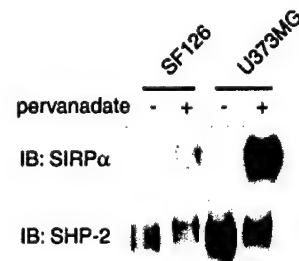


Fig. 6. Association of SHP-2 and signal regulatory protein (SIRP) α in astrocytoma cell lines. Lysates from the SF126 or U373MG cell lines at rest (lanes 1 and 3) or nonspecifically stimulated with pervanadate (lanes 2 and 4) were subjected to immunoprecipitation with a commercial antiserum against SHP-2 and then subjected to Western blot analysis using the 6.1 anti-SIRP α antibody (IB: SIRP α). Blots with an isotype-matched control antibody did not reveal bands (data not shown). Blots were subsequently stripped and reprobed with a monoclonal anti-SHP-2 antibody (IB: SHP-2).

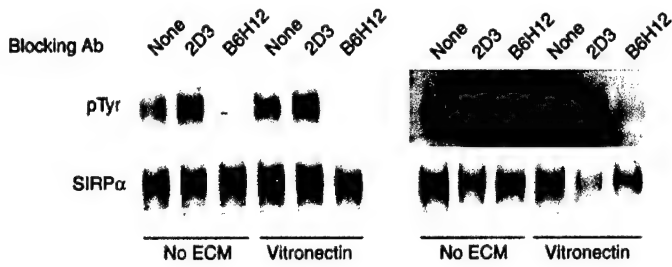


Fig. 7. Phosphorylation of signal regulatory protein (SIRP) α on cell aggregation or adhesion. Experiments were performed using 5×10^6 SF126 (left panels) or U373MG (right panels) cells resuspended in serum-free media and plated on standard uncoated tissue culture plates (No ECM) or vitronectin-coated plates (Vitronectin). Appropriate blocking antibody was added [either isotype-matched control (None), nonblocking anti-CD47 (2D3), or blocking anti-CD47 (B6H12)]. Cells were incubated overnight, and cell lysates were prepared and analyzed for SIRP α phosphorylation by immunoprecipitation and Western blotting as described in "Materials and Methods." The blot was then reprobed with a biotinylated anti-SIRP antibody and detected as described in "Materials and Methods." pTyr, phosphotyrosine.

the 6.1 anti-SIRP antibody (data not shown). Although CD47 is a known ligand for SIRP α , this is the first demonstration that CD47 induces phosphorylation of SIRP α . These results indicate that the phosphorylation of SIRP α requires an interaction with CD47; simple aggregation of cells is insufficient to cause SIRP α phosphorylation if the SIRP α -CD47 interaction is blocked. We also observed that simple adhesion to an extracellular matrix, in this case vitronectin, was

insufficient to cause phosphorylation of SIRP α when the SIRP α -CD47 interaction is blocked.

SIRPs and CD47 Are Expressed on Primary Brain Tumor Specimens. Although SIRP α is known to be expressed in the developing nervous system, in adult animals it is only expressed in limited regions of the central nervous system (11, 12, 14). Having established the expression of SIRPs on astrocytoma cell lines, we next examined the expression of SIRPs on samples from brain biopsies performed on patients with grade 4 astrocytomas. Immunohistochemistry was performed on frozen specimens because the 6.1 anti-SIRP antibody did not react with formalin-fixed, or paraffin-embedded samples. All specimens were verified to be from high-grade astrocytomas by WHO definitions (53). Slides were read in a blinded fashion by a neuropathologist (E. J. H.) who graded the degree of staining on an arbitrary scale from 0 to 4, and estimated the percentage of cells staining positive. Representative immunohistochemical sections are shown in Fig. 8, and complete results for all 10 samples are given in Table 1. In total, 7 of the 10 sections had detectable staining for SIRPs compared with staining by an isotype-matched control antibody. Control staining was never graded above 1+ (Fig. 8, compare middle panels with left panels), and never showed greater than 0–25% of cells positive. Some tumors expressed SIRPs quite strongly on all cells (sample SF3765), whereas others expressed it strongly on a limited numbers of cells (sample SF3911). Others expressed SIRPs weakly (sample SF4192), and others were completely negative (sample SF4393).

The sections of brain tumors were also examined by immunohis-

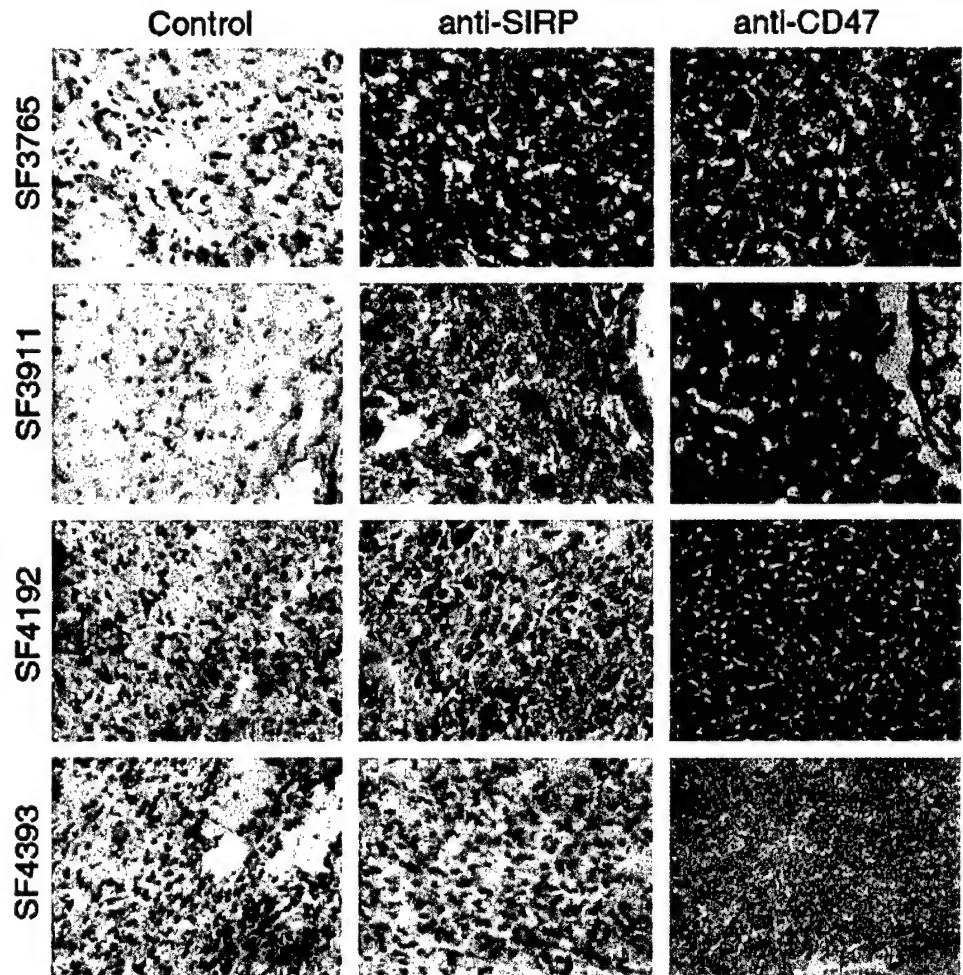


Fig. 8. Immunohistochemistry of astrocytoma specimens. Frozen sections from primary brain biopsies were analyzed by immunohistochemistry against signal regulatory protein α or CD47 and counterstained with hematoxylin. Four representative samples are shown. Staining with an isotype-matched control antibody is shown on the left panels, staining with anti-signal regulatory protein is shown on the middle panels, and staining for CD47 is shown on the right panels.

Table 1 Immunohistochemistry results

Frozen sections of 10 glioblastoma biopsies were stained with an isotype-matched control mAb^a (control), an anti-SIRP mAb (6.1), or an anti-CD47 mAb (B6H12) as described in "Materials and Methods." Slides were scored for the percentage of positive cells and intensity of staining in a blinded fashion.

Sample no.	Antibody	% positive	Intensity
3765	Control	0-25%	1+
	Anti-SIRP	75-100%	4+
	Anti-CD47	100%	2+
3910	Control	0-25%	1+
	Anti-SIRP	25-50%	2+
	Anti-CD47	75-100%	1+
3911	Control	0-25%	0-1+
	Anti-SIRP	25-50%	2+
	Anti-CD47	75-100%	1+
3932	Control	0-25%	1+
	Anti-SIRP	25-50%	1+
	Anti-CD47	0%	0+
4127	Control	0-25%	1+
	Anti-SIRP	75-100%	2+
	Anti-CD47	75-100%	2-3+
4192	Control	0%	0+
	Anti-SIRP	50-75%	1+
	Anti-CD47	75-100%	1+
4196	Control	0-25%	1+
	Anti-SIRP	50-75%	2+
	Anti-CD47	75-100%	2+
4209	Control	0%	0+
	Anti-SIRP	0-25%	1+
	Anti-CD47	75-100%	1+
4237	Control	0-25%	1+
	Anti-SIRP	0-25%	1+
	Anti-CD47	100%	3-4+
4393	Control	0%	0+
	Anti-SIRP	0%	0+
	Anti-CD47	0%	0+

^a mAb, Monoclonal antibody; SIRP, signal regulatory protein.

tochemistry for expression of CD47. Although all sections stained strongly with the anti-CD47 mAb and not with control mAb, there was occasionally background staining of areas without viable cells so that we were confident of CD47 expression on tumor cells in only 8 of the 10 specimens. In summary, both primary brain tumors and glioblastoma-derived cell lines frequently express SIRPs, and most, if not all, cell lines and frozen tumors express the SIRP α ligand CD47.

DISCUSSION

In this report, we first provide evidence for expression of SIRP α on both astrocytoma cell lines and brain tumor biopsies. The majority of cell lines and tumors express SIRP α , although levels of expression vary considerably. As assessed by studies of tumor cell lines, most, if not all, of the SIRP α expressed on astrocytomas is identical in sequence to SIRP α 1 (3, 4). Furthermore, Northern blotting demonstrates the characteristic pattern for SIRP α 1 in all cell lines that are positive for SIRP α expression, although transcripts are also expressed in cell lines without detectable SIRP α 1 on the cell surface. Second, we have shown that SIRP α in these astrocytoma cell lines is functional in that it can bind its ligand CD47, is phosphorylated in a CD47-dependent manner, and associates with SHP-2 on phosphorylation. To the best of our knowledge, this is the first demonstration that SIRP α is expressed on astrocytoma cell lines or on primary brain tumors.

Proximal signaling events in SIRP α activation include phosphoryl-

ation on tyrosine residues on ITIMs. This phosphorylation can be induced by several stimuli, although to our knowledge phosphorylation of SIRP α by CD47 fusion proteins has not been directly examined (7, 20, 21, 54). Our studies indicate that SIRP α phosphorylation can be induced by interaction with CD47, and adhesion to vitronectin is insufficient to induce SIRP α phosphorylation without the recognition of CD47. We were unable to demonstrate SIRP α phosphorylation with our CD47Fc fusion protein (data not shown). Similarly, we were unable to observe SIRP α phosphorylation upon cross-linking with our 6.1 mAb, which instead blocked SIRP α phosphorylation in our aggregation assay (data not shown). Thus, the requirements for phosphorylation of SIRP α appear to extend beyond mere cross-linking of SIRP α but are met when astrocytoma cells expressing both SIRP α and CD47 aggregate.

Several reports suggest that cell-cell adhesion can be entirely mediated by a SIRP α -CD47 interaction (10, 17, 42); however, adhesion of monocytes to endothelial cells is not dependent on the SIRP α -CD47 interaction (37). In our studies, homotypic adhesion of astrocytoma cell lines to each other was not affected by the SIRP α -CD47 interaction because aggregation was unaffected by blocking the SIRP α -CD47 interaction, and cell lines aggregated to the same extent regardless of their expression of SIRP α (data not shown). Therefore, SIRP α phosphorylation on astrocytoma cell lines is not dependent on cell aggregation *per se* but only on the interaction of SIRP α and CD47.

Our findings may be important with regard to several aspects of astrocytoma behavior. SIRP α is known to be involved in cytoskeletal reorganization and cell motility in transfected cells (27, 38) as well as neurite outgrowth (13, 14, 41). In addition, multiple reports have established an important role for SIRP α in the migration and adhesion of macrophages to various substrates (10, 37, 42, 55) as well as in neutrophil chemotaxis (21, 28). Astrocytomas are characterized by extensive infiltration and a striking ability to metastasize locally, processes that are known to be integrin-dependent (reviewed in Refs. 56 and 57). Thus, the presence of SIRP α might influence the ability of tumor cells to migrate and invade the central nervous system. Indeed, prior studies have reported that transfection of SIRP α into the glioblastoma cell line U87MG results in defective cell spreading and migration (40). However, it is likely that SIRP α affects cell motility and adhesion in complex and dynamic ways. For example, fibroblasts derived from mice homozygous for expression of a mutant SIRP α , missing most of the cytoplasmic tail, exhibit accelerated initial adhesion to fibronectin but defective subsequent polarized extension and migration (38). In addition, the role of SIRP α in the regulation of cell biology may be different between transfected cells and cells that express SIRP α endogenously. We have thus far not observed any clear correlations between growth characteristics of astrocytoma cell lines and SIRP α expression, and no consistent differences in morphology are evident between cell lines that express SIRP α and those that do not. Studies on these cell lines are currently under way to examine the effect of endogenous SIRP α expression on more subtle tumor characteristics, such as cytoskeletal reorganization, cell motility, and adhesion.

SIRP α may also affect the response of astrocytomas to mitogenic growth signals. Most reports have shown that expression of SIRP α inhibits signaling through growth factor receptors, including those with intrinsic tyrosine kinase activity [EGF receptor (4, 33), platelet-derived growth factor receptor (4), and insulin receptor (31)], receptors that recruit cytoplasmic tyrosine kinases [growth hormone receptor (29, 30, 58) and IgE Fc receptor (59)], and G protein-coupled receptors [lysophosphatidic acid receptor (36)]. However, one group has shown that SIRP α enhances MAPK phosphorylation through the insulin receptor in NIH3T3 or Rat1 cells (32). Other groups have

shown no effect of SIRP α expression on specific aspects of the MAPK signaling cascade, such as EGF-induced MAPK phosphorylation (40), lysophosphatidic acid stimulation of MAPK (36), or fibronectin-mediated phosphorylation of MAPK (26). These experiments all examined cells overexpressing wild-type SIRP α or mutant SIRP α , which could potentially complicate interpretation. We are initiating experiments to examine these effects in astrocytoma lines.

It was important for us to verify the identity of the SIRP expressed on these cell lines because isoforms of SIRP α have been described, and they differ predominantly in the membrane-distal immunoglobulin (V) domain (4). Sequencing of the human genome has thus far revealed only two genes for SIRP α receptors. The first is encoded by eight exons on chromosome 20p13, telomeric to genes for SIRP β receptors (25, 60). An orthologous gene is encoded on mouse chromosome 2 (61). It appears likely that at least two cDNA variants for SIRP α (*SIRP α 1* and *SIRP α 2*) are alleles of this locus. These two genes vary almost exclusively in their first immunoglobulin-like domain. The sequence in the NCBI database encodes the *SIRP α 2* sequence, and there is no other gene or alternate exon in the NCBI database that encodes *SIRP α 1*.⁷ There is, however, a second SIRP α gene on chromosome 22, which is encoded as a single exon.⁷ The open reading frame of this gene is uninterrupted by stop codons, and it appears to encode the *SIRP α 3* cDNA. In the two cell lines that we examined in detail (SF126 and U373MG), all but one of the sequences recovered by PCR has matched *SIRP α 1*. Only one PCR clone matched *SIRP α 2*, and in no cases have we identified expression of *SIRP α 3*.

Prior groups have reported alternately spliced forms of *SIRP α* resulting in a single extracellular immunoglobulin V domain (11, 25). In the present study, most cDNAs cloned by PCR included all immunoglobulin domains, but sequences corresponding to the single immunoglobulin domain splice variant were also detected. Northern blotting, however, showed this variant to be a low abundance transcript. These results are similar to those of Comu *et al.* (11), who described several minor bands that may correspond to alternatively spliced *SIRP α* transcripts in mouse brain total RNA. Characterization of the murine *SIRP α* locus has revealed several other potential splice variants as detected by blotting or direct sequencing (11, 25, 52). In our analysis, these alternative forms were not seen, although other potential novel splice variants were detected at low frequency. These truncated sequences are likely splice variants because the deleted regions correspond exactly to the predicted exonic structure of *SIRP α* (25), and all sequences are in-frame gene products. The products of the splice variants are not abundant by Northern blotting, but additional studies may reveal that they have functional significance.

In analyzing the expression of SIRP α transcripts on astrocytoma cell lines by Northern blotting, we discovered that eight of nine cell lines expressed mRNA for SIRP α , although only five of these expressed detectable levels of cell surface protein by flow cytometry. We have not detected intracellular SIRP α protein by Western blotting in cells that do not express surface SIRP α (data not shown). Thus, regulation of SIRP α expression probably involves not only transcriptional but also posttranscriptional events. Alternatively, some cell lines may express SIRP α mRNA with mutations that render the SIRP α protein nonreactive to our mAb, or posttranslational modifications of SIRP α may render a gene product not detectable by our 6.1 mAb. This latter possibility seems unlikely because our 6.1 mAb recognizes deglycosylated SIRP α protein (Fig. 2), which implies that it recognizes an epitope on the core polypeptide. Also, we have shown that the degree of SIRP α surface expression, as assayed by the 6.1

mAb, corresponds to the degree of binding to the CD47Fc fusion protein (Fig. 5). Interestingly, Machida *et al.* (45) have reported that transfection of 3T3 fibroblasts with v-src inhibits SIRP α expression at the transcriptional level, and a recent abstract (62) finds that interfering with the EGF receptor signaling pathway in U87MG or U373MG cells by transfection of a dominant negative EGF receptor mutant can up-regulate SIRP α mRNA levels. We are unaware of any other reports that specifically examine regulation of SIRP α expression at the transcriptional level, although some reports hypothesize that expansion of CCA trinucleotide repeats found in the 3'-untranslated region of the SIRP α gene may be responsible for altered expression in disease states (52, 60). Nevertheless, the caveat remains that mRNA levels may not directly correlate with SIRP α cell surface expression. This may have consequences in experiments using quantitative PCR or cDNA microarrays to examine differential gene expression of SIRP α .

In our attempts to identify the SIRPs expressed on astrocytoma cell lines, we discovered that, although the primary DNA sequence was identical to SIRP α 1, the apparent molecular weight of the SIRP α 1 protein was slightly less than that of SIRP α 1 expressed in CHO cells, due to differences in glycosylation. Reduced glycosylation of SIRP α in neuronal cells has been noted previously in rodent tissues (8, 23, 63), although its functional significance is unknown. In one report, a nonglycosylated SIRP α 1-gluathione *S*-transferase fusion protein produced in bacteria was capable of inhibiting SIRP α 1-mediated macrophage fusion, implying that at least one activity of SIRP α 1 is not dependent on glycosylation (24). In contrast, the binding affinity of a SIRP α 1-Fc fusion protein to tissue sections was changed when the fusion protein was produced in cells deficient in galactosylation (23), arguing that SIRP α 1 binding to CD47 is altered on undergalactosylation. In our studies, SIRP α 1 expressed by astrocytoma cell lines demonstrated this reduced glycosylation, yet it was still capable of binding to a CD47Fc fusion protein. Detection of binding required coincubation with the 2D3 antibody against CD47, which increases the avidity of the CD47-SIRP α interaction.⁷ CD47 has several isoforms that are differentially expressed in various tissues (64), and this diversity may contribute to the variable binding by CD47 to SIRP α that has been observed by others (17, 23). In addition, a SIRP α 1 fusion protein binds to CD47 on resting CD4⁺ or CD8⁺ T cells, but binding decreases on stimulation of the T cells with concanavalinA, despite equivalent expression of CD47 on the T cells (65). Thus, the affinity of the CD47-SIRP α interaction may vary depending on the biological context and may not simply reflect expression of the molecules *per se*. It is interesting to consider the possibility that SIRP α 1 expressed on astrocytomas may interact with CD47 on T cells, altering the immune response to these tumors.

Although SIRP α 1 is expressed on astrocytomas and other tumors, its role in oncogenesis is unknown. Our results show that SIRP α is expressed in malignant astrocytes, whereas mouse studies have shown that normal astrocytes do not express SIRP α (7). Our studies demonstrate that levels of SIRP α expression vary considerably in different tumors. It will be of interest to test the correlation between SIRP α expression and outcomes in patients with astrocytomas. Furthermore, the ability of SIRP α to modulate multiple pathways critical to the malignant behavior of tumors may offer new targets for therapeutic intervention in the treatment of malignancies in general and malignant astrocytomas in particular.

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⁷ T. Chen and W. Seaman, unpublished observations.

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